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FORM PTO-1390 (REV. 11-2000) U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE

TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371

ATTORNEY 'S DOCKET NUMBER	
6580-270	
U.S. APPLICATION NO. (If known, see 37 CFR 1.5)	
09/926375	

INTERNATIONAL APPLICATION NO.	INTERNATIONAL FILING DATE	PRIORITY DATE CLAIMED
PCT/CA00/00430	April 20, 2000	April 23, 1999

TITLE OF INVENTION: **TRANSGENIC ANIMALS EXPRESSING SALIVARY PROTEINS**

APPLICANT(S) FOR DO/EO/US: **Cecil W. Forsberg, Serguei Golovan, John P. Phillips**

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☑ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. ☐ This is an express request to begin national examination procedures (35 U.S.C. 371(f)). The submission must include items (5), (6), (9) and (21) indicated below.
4. ☐ The US has been elected by the expiration of 19 months from the priority date (Article 31).
5. ☑ A copy of the International Application as filed (35 U.S.C. 371(c)(2))
   a. ☐ is attached hereto (required only if not communicated by the International Bureau).
   b. ☑ has been communicated by the International Bureau.
   c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US).
6. ☐ An English language translation of the International Application as filed (35 U.S.C. 371(c)(2)).
   a. ☐ is attached hereto.
   b. ☐ has been previously submitted under 35 U.S.C. 154(d)(4).
7. ☑ Amendments to the claims of the International Aplication under PCT Article 19 (35 U.S.C. 371(c)(3))
   a. ☐ are attached hereto (required only if not communicated by the International Bureau).
   b. ☐ have been communicated by the International Bureau.
   c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
   d. ☑ have not been made and will not be made.
8. ☐ An English language translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371 (c)(3)).
9. ☐ An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)).
10. ☐ An English lanugage translation of the annexes of the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).

Items 11 to 20 below concern document(s) or information included:

11. ☐ An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
12. ☐ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
13. ☑ A FIRST preliminary amendment.
14. ☐ A SECOND or SUBSEQUENT preliminary amendment.
15. ☐ A substitute specification.
16. ☐ A change of power of attorney and/or address letter.
17. ☐ A computer-readable form of the sequence listing in accordance with PCT Rule 13ter.2 and 35 U.S.C. 1.821 - 1.825.
18. ☐ A second copy of the published international application under 35 U.S.C. 154(d)(4).
19. ☐ A second copy of the English language translation of the international application under 35 U.S.C. 154(d)(4).
20. ☐ Other items or information:

U.S. APPLICATION NO. (If known, see 37 CFR 1.5)	INTERNATIONAL APPLICATION NO.	ATTORNEY'S DOCKET NUMBER
09/926375	PCT/CA00/00430	6580-270

	CALCULATIONS PTO USE ONLY	
21. ☑ The following fees are submitted:		
BASIC NATIONAL FEE (37 CFR 1.492 (a) (1) - (5)):		
Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO **$1000.00**		
International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO **$860.00**		
International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2)) paid to USPTO **$710.00**		
International preliminary examination fee (37 CFR 1.482) paid to USPTO but all claims did not satisfy provisions of PCT Article 33(1)-(4) **$690.00**		
International preliminary examination fee (37 CFR 1.482) paid to USPTO and all claims satisfied provisions of PCT Article 33(1)-(4) **$100.00**		
ENTER APPROPRIATE BASIC FEE AMOUNT =	$ **890.00**	
Surcharge of **$130.00** for furnishing the oath or declaration later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492(e)).	$	

CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE		
Total claims	- 20 =		x **$18.00**	$	
Independent claims	- 3 =		x **$80.00**	$	
MULTIPLE DEPENDENT CLAIM(S) (if applicable)			+ **$270.00**	$	
TOTAL OF ABOVE CALCULATIONS =				$ **890.00**	
☐ Applicant claims small entity status. See 37 CFR 1.27. The fees indicated above are reduced by 1/2. +				$	
SUBTOTAL =				$ **890.00**	
Processing fee of **$130.00** for furnishing the English translation later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492(f)).				$	
TOTAL NATIONAL FEE =				$ **890.00**	
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). **$40.00** per property +				$	
TOTAL FEES ENCLOSED =				$ **890.00**	
				Amount to be refunded:	$
				charged:	$

a. ☑ A check in the amount of $ **890.00** to cover the above fees is enclosed.

b. ☐ Please charge my Deposit Account No. _____ in the amount of $ _____ to cover the above fees. A duplicate copy of this sheet is enclosed.

c. ☑ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. **022095** . A duplicate copy of this sheet is enclosed.

d. ☐ Fees are to be charged to a credit card. **WARNING:** Information on this form may become public. **Credit card information should not be included on this form.** Provide credit card information and authorization on PTO-2038.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137 (a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO:

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M. Gravelle
SIGNATURE

MICHELINE GRAVELLE
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40,261
REGISTRATION NUMBER

Bereskin & Parr

Barristers and Solicitors/Patent and Trade Mark Agents
Practice Restricted to Intellectual Property Law

October 22, 2001

Micheline Gravelle B.Sc., M.Sc. (Immunol.)
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Your Reference: n/a
Our Reference: 6580-270

Commissioner for Patents and Trademarks
Washington, D.C. 20231
U.S.A.

Dear Sirs:

Re: PRELIMINARY AMENDMENT
United States National Phase Entry of PCT/CA00/00430
Entitled: Transgenic Animals Expressing Salivary Proteins
Inventors: Cecil W. Forsberg, Serguei Golovan, John P. Phillips

We are simultaneously entering national phase in the United States for PCT/CA00/00430. The present letter is to file a Preliminary Amendment to the application. Please amend the application as follows:

In the Claims:

Please amend claims 9, 10, 11, 12, 15 and 53 as follows:

9. (Amended) The animal of claim 1 wherein said animal is a pig.

10. (Amended) The animal of claim 1 wherein said protein is a phytase.

11. (Amended) The animal of claim 1 wherein said animal is a pig, said protein is a phytase and said first regulatory sequence comprises a parotid secretory protein (PSP) promoter/enhancer or a proline-rich protein (PRP) promoter/enhancer.

12. (Amended) The animal of claim 1 wherein said transgene construct comprises a nucleic acid sequence according to SEQ ID NO:3, SEQ ID NO:5; or SEQ ID NO:7.

15. (Amended) The animal of claim 13 wherein said transgene is operably linked to a first regulatory sequence for salivary gland specific expression of said phytase.

53. (Amended) A host cell transfected with molecule according to claim 44.

REMARKS

By the present amendment, claims 9, 10, 11, 12, 15 and 53 have been amended in order to delete multiple dependencies. The Preliminary Amendment does not contain new matter.

Entry of the above preliminary amendment is respectfully requested. Please calculate the claim fee for the application once the amendment has been entered.

Attached hereto is a marked-up version of the changes made to the claims by the current amendment. The attached page is captioned "Version with markings to show changes made".

Respectfully submitted,

Cecil W. Forsberg et al.

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VERSION WITH MARKINGS TO SHOW CHANGES MADE

In the Claims:

Claims 9, 10, 11, 12, 15 and 53 have been amended as follows:

9. (Amended) The animal of [any one of] claim[s] 1 [to 8] wherein said animal is a pig.

10. (Amended) The animal of [any one of] claim[s] 1 [to 9] wherein said protein is a phytase.

11. (Amended) The animal of [any one of] claim[s] 1 [to10] wherein said animal is a pig, said protein is a phytase and said first regulatory sequence comprises a parotid secretory protein (PSP) promoter/enhancer or a proline-rich protein (PRP) promoter/enhancer.

12. (Amended) The animal of [any one of] claim[s] 1 [to 11] wherein said transgene construct comprises a nucleic acid sequence according to SEQ ID NO:3, SEQ ID NO:5; or SEQ ID NO:7.

15. (Amended) The animal of claim 13 [or 14] wherein said transgene is operably linked to a first regulatory sequence for salivary gland specific expression of said phytase.

53. (Amended) A host cell transfected with molecule according to [any one of] claim[s] 44 [to 48].

TRANSGENIC ANIMALS EXPRESSING SALIVARY PROTEINS

FIELD OF THE INVENTION

The present invention relates to transgenic animals and, more specifically, to animals genetically modified to express a desired protein.

BACKGROUND OF THE INVENTION

Phosphorus is an essential element for the growth of all organisms. In livestock production, phosphorus deficiency has been described as the most prevalent mineral deficiency throughout the world and feed must often be supplemented with inorganic phosphorus in order to obtain desired growth performance of monogastric animals (e.g. pigs, poultry etc.).

Phytic acid, or phytate, (*myo*-inositol 1,2,3,4,5,6-hexakis dihydrogen phosphate) is a major storage form of phosphorus in cereals and legumes, representing 18% to 88% of the total phosphorus content (Reddy *et al.* 1982). The enzyme phytase (*myo*-inositol hexakisphosphate phosphohydrolase) belongs to the group of phosphoric monoester hydrolases: it catalyzes the hydrolysis of phytate (*myo*-inositol hexakis phosphate) to inorganic monophosphate and lower phosphoric esters of *myo*-inositol or, in some cases, free *myo*-inositol. Phytases are classified either as 3-phytases or 6-phytases based on the first phosphate group attacked by the enzyme. 3-phytase is typical for microorganisms and 6-phytase for plants (Cosgrove, 1980).

Phytase is either absent or present at a very low levels in monogastric animals (Bitar and Reinhold 1972; Iqbal *et al.* 1994). Consequently, dietary phytate is not digested or absorbed from the small intestine and instead is concentrated in fecal material, thereby contributing to phosphorus pollution in areas of intensive livestock production. Runoff from animal farms leads to contamination of rivers and streams. Such runoff has resulted in rapid drops in the oxygen concentration in rivers and streams due to excessive algal growth in water, which, in turn, has led to an increase in the mortality rate of fish and existing flora and fauna. This is becoming a global problem as pig and poultry production is increased (Miner 1999;Mallin 2000). Furthermore, phytic acid is viewed as an anti-nutritional factor because it interacts with essential dietary minerals and proteins limiting the nutritional values of cereals and legumes in man and animals (Harland and Morris 1995).

For the above reasons, various attempts have been made to enable animals to utilize available phytate in feed. Such attempts have included production of low phytate plants (Abelson 1999), addition of phytase to the animal feed (Simons *et al.* 1990) (Stahl *et al.* 1999) or transformation of the fodder plants to produce the required phytase (Pen *et al.* 1993, Verwoerd *et al.* 1995). A combination of these options, the feeding of phytase to poultry receiving low phytate corn has also been tested (Huff *et al.* 1998). However, these solutions increase the cost of animal production. Also because phytase is an enzyme, it is susceptible to inactivation by heat and moisture and is generally unstable at the high temperatures used for feed pelleting.

The primary phytase used for supplementing animal feeds is from *Asperigillus* sp.; however, phytases are produced by a large number of plants and microorganisms (Wodzinski and Ullah 1996) (Dvorakova 1998). A phytase produced by *Escherichia coli* has been reported to exhibit the highest activity of those reported (Wodzinski and Ullah 1996). This phytase from *E. coli* was initially cloned as an acid phosphatase gene that was designated *APPA* (Dassa *et al.* 1990). Greiner et al. (1991; 1993) purified phytase from *E. coli* and reported that some of the kinetic properties of the acid phosphatase activity of the native phytase of *E. coli* were similar to those of the *APPA*-encoded acid phosphatase. However, the authors did not clone the phytase gene to prove that it was identical to *APPA* gene. We have subsequently cloned, overexpressed and characterized *APPA* gene, and shown that the *E. coli* gene *APPA* codes for a bifunctional enzyme exhibiting both phytase and acid phosphatase activities (Golovan *et al.* 2000). Phytases exhibit phosphatase activity, however the relative activities differ widely among enzymes (Wodzinski and Ullah 1996).

Therefore, there is a need for an improved method of allowing access by animals to phytase so as to enable efficient phytate metabolism and, thereby reducing phosphate pollution.

In the field of protein production using recombinant methods, one of the associated problems relates to the lack of required glycosylation. Therefore, a method of producing such glycoproteins is also needed.

SUMMARY OF THE INVENTION

In one embodiment, the invention provides a transgenic non-human animal that carries in the genome of its somatic and/or germ cells a nucleic acid sequence including a heterologous transgene construct, the construct including a trangene encoding a protein, the

transgene being operably linked to a first regulatory sequence for salivary gland specific expression of the protein.

In another embodiment, the invention provides a transgenic non-human animal that carries in the genome of its somatic and/or germ cells a nucleic acid sequence including a heterologous transgene construct, the construct including a trangene encoding phytase or a homologue thereof.

In yet another embodiment, the invention provides a method of expressing a protein, the method comprising the steps of:

a) introducing a transgene construct into a non-human animal embryo such that a non-human transgenic animal that develops from the embryo has a genome that comprises the transgene construct, wherein the transgene construct comprises:

i) a transgene encoding the protein, and

ii) at least one regulatory sequence for gastrointestinal tract specific expression of the protein,

b) transferring the embryo to a foster female; and,

c) developing the embryo into the transgenic animal

wherein the transgene is produced in the gastrointestinal tract of the animal.

In a further embodiment, the invention provides a transgenic animal adapted for expressing a protein according to the above method. The invention also provides for the progeny of such animal.

In another embodiment, the invention provides a process for producing a protein comprising the steps of:

a) obtaining saliva containing the protein from a non-human transgenic animal, the animal containing within its genome a transgene construct, wherein the transgene construct comprises:

i) a transgene encoding the protein, and

ii) at least one regulatory sequence for salivary gland specific expression of the protein, and

extracting the protein from the saliva.

In a further embodiment, the invention provides a method for expressing a phytase or a homologue thereof in a non-human animal, the method comprising:

a) constructing a nucleic acid sequence including a transgene construct comprising:

i) a transgene encoding the phytase or a homologue thereof, and

ii) at least one regulatory sequence for gastrointestinal tract specific expression of the protein, and

b) transfecting the animal with the nucleic acid sequence;

whereby the animal carries within the genome of its somatic and/or germ cells the transgene construct and wherein the animal expresses the phytase or a homologue thereof in its gastrointestinal tract.

In another embodiment the invention provides a nucleic acid molecule comprising a nucleic acid sequence including a gene encoding a protein, the gene being operably linked to at least one regulatory sequence for gastrointestinal tract specific expression of the protein.

In another embodiment the invention provides an antibody specific to the protein expressed by the above nucleic acid sequence and a test kit for immunologically detecting such protein. The invention also provides for hybridomas secreting such antibodies.

In another embodiment the invention provides cells that are transfected with the above nucleic acid sequence.

In another embodiment, the invention provides a method for producing a protein molecule comprising a glycosylated protein secreted in the saliva that exhibits a novel physiological activity. One example of such an activity is phytase.

BRIEF DESCRIPTION OF THE DRAWINGS

These and other features of the preferred embodiments of the invention will become more apparent in the following detailed description in which reference is made to the appended drawings wherein:

Figure 1 is a schematic diagram representing a method for producing the gene construct of the present invention containing the inducible proline-rich protein (PRP) promoter/enhancer. More specifically, Figure 1 is a schematic diagram illustrating the steps in the construction of the transgenes R15/APPA+intron and R15/APPA used for the generation of transgenic mice.

Figure 2 is a schematic diagram representing a method for producing the gene construct of the present invention containing the SV40 promoter. More specifically, Figure 2 is a schematic diagram illustrating the steps in construction of the plasmid containing the transgene SV40/APPA+intron that was introduced by transfection into mammalian cell lines.

Figure 3 is a schematic diagram representing a method for producing the gene construct of the present invention containing the constitutive parotid secretory protein (PSP) promoter/enhancer. More specifically, Figure 3 is a schematic diagram illustrating the steps

in construction of the transgenes Lama2/APPA that codes for the native AppA phytase and the Lama2/PSP/APPA that codes for the AppA phytase with the PSP signal peptide sequence.

Figure 4 is a schematic diagram of the Lama2-APPA plasmid containing the APPA transgene.

Figure 5 illustrates the nucleic acid sequence of the Lama2/APPA plasmid containing the *E. coli APPA* gene (SEQ ID NO: 1).

Figure 6 illustrates the PCR results for transformed mice. More specifically, figure 6 is a picture of an agarose gel illustrating APPA PCR products from genomic tail DNA of third generation offspring from the transgenic female founder mouse 3-1 generated using the *Xho*1 and *Not*1 fragment of the Lama2/APPA construct. A second generation phytase gene positive male was crossed with each of two phytase positive transgenic females 9f and 11f (Table 3). From litter 18m x 9f offspring 3, 4, 5 & 6 are PCR positive and from litter 18m x 11f offspring 2 and 3 are PCR positive. **Std** is the oligonucleotide standard and the numbers on the left are the bp sizes of the standard. **Lane C** is a negative control reaction mixture that lacks a DNA template and ***appA*** is a positive control containing an amplified segment of the phytase gene. The primers used were APPA-UP2 and APPA-KPN.

Figure 7 illustrates the PCR results for transformed founder pigs. More specifically, Figure 7 is a picture of an agarose gel illustrating phytase gene PCR products and β-globin PCR products from genomic tail DNA of five founder piglets from litter 167. **Std** is a 1 kb ladder. **Lane 2** using the phytase primer set is positive for the phytase gene, and all of the samples were positive for the β-globin gene. **Lane C** is a negative control not containing template DNA. The phytase transgene primer set included APPA-UP2 and APPA-KPN gave an expected fragment size of 750 bp. The primer set for the β-globin gene included PIG-BGF and PIG-BRG gives an expected fragment size of 207 bp.

Figure 8 illustrates the PCR results for transgene rearrangement tests. More specifically, Figure 8 is a picture of an agarose gel showing the PCR products of four separate primer sets used to amplify different segments of the transgene introduced into pig 167-02. The **Std** contained a kilobase DNA ladder. The primers used included **lane 1,** APPA-UP2 and APPA-KPN (750 bp); **lane 2,** APPA –MATURE and APPA-KPN (1235 bp); **lane 3** APPA MATURE and APPA-DOWN2 (608 bp); **lane 4,** PIG-BGF and PIG-BGR (207 bp). **lane 5,** a negative control without DNA template added; **lane 6,** the *appA* gene & primers APPA-UP2 and APPA-KPN. The numbers on the left indicate the sizes of the bands in the standard. No PCR products were detected in the absence of either DNA template or primers.

Figure 9 illustrates weight and salivary phytase activity of the transgenic boar 167-02 and average weight of the pen-mates at intervals during growth. Symbols: Weight of 167-02, ●; Average weight ± SD of four penmates, ▲; phytase activity of 167-02, ■; Phytase specific activity, □. Arrows indicate sampling for fecal phosphorus concentration.

Figure 10 illustrates weight and salivary phytase activity of the transgenic boar 282-02 and average weight of the pen-mates at intervals during growth. Symbols: Weight of 282-02, ●; Average weight ± SD of five penmates, ▲; phytase activity of 282-02, ■; Phytase specific activity, □. Arrows indicate sampling for fecal phosphorus concentration.

Figure 11 illustrates weight and salivary phytase activity of the transgenic boar 282-04 and average weight of the pen-mates at intervals during growth. Symbols: Weight of 282-04, ●; Average weight ± SD of five penmates, ▲; phytase activity of 282-04, ■; Phytase specific activity, □. Arrows indicate sampling for fecal phosphorus concentration.

Figure 12 illustrates weight and salivary phytase activity of the transgenic boar 405-02 and average weight of the pen-mates at intervals during growth. Symbols: Weight of 405-02, ●; Average weight ± SD of four penmates, ▲; phytase activity of 405-02, ■; Phytase specific activity, □. Arrows indicate sampling for fecal phosphorus concentration.

Figure 13 illustrates weight and salivary phytase activity of the transgenic boar 421-06 and average weight of the pen-mates at intervals during growth. Symbols: Weight of 421-06, ●; Average weight ± SD of four penmates, ▲; phytase activity of 421-06, ■; Phytase specific activity, □. Arrows indicate sampling for fecal phosphorus concentration.

Figure 14 illustrates the PCR results of first generation pigs. More specifically, Figure 14 is a picture of an agarose gel showing the PCR analysis of eight liter 154 piglets. The phytase transgenic boar 167-02 was used to breed a non-transgenic female. **Std**, 100 bp ladder, numbers on left are the sizes of the fragments in each band in bp; **lane 167-02**, DNA from boar 167-02 1, DNA from 167-02; **lane C**, is a lane without added DNA; **lanes 1-8**, are amplified DNA inserts from each of the offspring piglets of the litter. Phytase primers were Lama-UP and APPA-DOWN4. β-globin primers were PIG-BGF and PIG-BGR.

Figure 15 illustrates a sodium dodecylsulfate gel stained with silver demonstrating the sizes of the *E. coli* produced APPA phytase and the APPA phytase produced by the pig and a demonstration that the pig phytase is glycosylated. More specifically, Figure 15 is a picture of a sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) profile of the purified AppA phytase produced in *E. coli* and the purified pig salivary phytase stained directly with silver (A) and a transfer from a similar SDS-PAGE gel transferred to

nitrocellulose and stained for glyoproteins (**B**). Creatinase is not glycosylated while transferring is glycosylated. The numbers on the left are the masses in of the molecular mass standards (**Std**) expressed in kDa.

Figure 15B is a picture of Western blot of the untreated pig AppA phytase and the same phytase after treatment with a combination of three deglycosylating enzymes. **Lane 1**, Purified AppA phytase produced in *E. coli* (untreated); **lane 2**, purified pig phytase (untreated); **lane 3**, purified pig phytase treated with the combination of deglycosylating enzymes including N-glycosidase F, O-glycosidase and neuraminidase.

Figure 16 illustrates a Western blot of the pig phytase and the *E. coli* produced APPA phytase using monoclonal antibodies directed to the APPA phytase documenting that they have homologous epitopes. More specifically, Figure 6 is a Western blot of the AppA phytase from pig saliva after various purification steps and of purified phytase produced in *E. coli*. A monoclonal antibody prepared against the *E. coli* phytase was used as the primary antibody for detection. **Lane 1**, saliva from non-transgenic pig 164-04; **lane** 2, saliva from transgenic pig 167-02; **Lane 3**, saliva fraction not bound to DEAE-Sepharose; **lane 4**, salivary phytase bound to DEAE-Sepharose and released with an NaCl gradient; **lane 5**, salivary phytase further purified by Chromatofocusing with a pH gradient of 4 to 7; **lane 6**, phytase purified from *E. coli*. The numbers on the left are the masses of molecular mass standards (not shown) expressed in kDa.

Figure 17 illustrates an SDS-Page of the *E. coli* APPA phytase, saliva samples from phytase negative and positive pigs and mice and a corresponding Western blot documenting that phytases from all three sources have homologous antigenic epitopes, but the animal phytases are larger than that produced in *E. coli*. More specifically, Figure 6 is a SDS-PAGE profile of the purified *E. coli* produced AppA phytase and the AppA phytases produced by pigs and mice stained with silver (A) and a Western blot of an identical set of protein samples (B). A polyclonal antibody prepared against the *E. coli* phytase was used as the primary antibody for detection. **Lane 1**, Purified AppA phytase produced in *E. coli*; **lane 2**, Saliva from a non-transgenic pig 164-01; **lane 3**, Saliva from a AppA producing transgenic pig 167-02; **lane 4**, Purified phytase from pig 167-02; **lane 5**, Saliva from a non-transgenic mouse; **lane 6**, Saliva from a transgenic mouse containing R15/APPA transgene induced with isoproterenol; **lane 7**, Saliva from a transgenic mouse containing the Lama/APPA transgene; **Std**, Molecular mass markers. The numbers on the left are the masses of molecular mass standards (not shown) expressed in kDa.

Figure 18 illustrates the nucleic acid sequence of the known segment of the R15/APPA + intron plasmid including the vector sequences of pBLCAT3 (SEQ ID NO:2).

Figure 19 illustrates the nucleic acid sequence of the known segment of the R15/APPA + intron transgene construct used for the generation of transgenic mice (SEQ ID NO:3).

Figure 20 illustrates the nucleic acid sequence of the known segment of the R15/APPA plasmid including the vector sequences of pBLCAT3 (SEQ ID NO:4).

Figure 21 illustrates the nucleic acid sequence of the known segment of the R15/APPA transgene construct used for the generation of transgenic mice (SEQ ID NO:5).

Figure 22 illustrates the nucleic acid sequence of the SV40/APPA + intron plasmid (SEQ ID NO:6).

Figure 23 illustrates the nucleic acid sequence of the Lama2/APPA transgene construct used for the generation of transgenic mice and transgenic pigs (SEQ ID NO: 7).

DESCRIPTION OF THE PREFERRED EMBODIMENTS

In the following description, a number of recombinant DNA technology terms are used. The following definitions have been provided in order to enable a clearer understanding of the specification and appended claims:

"Promoter" - a DNA sequence generally described as the 5' region of a gene and located proximal to the start codon. The transcription of an adjacent gene is initiated at the promoter region. If a promoter is an inducible promoter then the rate of transcription increases in response to an inducing agent. A constitutive promoter is one that initiates transcription of an adjacent gene without additional regulation.

"Operably Linked" - a nucleic acid sequence is "operably linked" when placed into a functional relationship with another nucleic acid sequence. For instance, a promoter or enhancer is "operably linked" to a coding sequence if the promoter causes the transcription of the sequence. Generally, operably linked means that the linked nucleic acid sequences are contiguous and, where it is necessary to join two protein coding regions, contiguous and in one reading frame.

"Phytase" - any protein that liberates phosphate from myo-inositolhexakis-phosphate or other inositol phosphates. Its catalytic capability may be limited to phytic acid or one of its salts, or it may show less specificity and hydrolyze a variety of phosphorylated compounds.

"Gene" - a DNA sequence that contains a template for an RNA polymerase and contains information needed for expressing a polypeptide or protein.

"Polynucleotide Molecule" - a polydeoxyribonucleic (DNA) acid molecule or a polyribonucleic acid (RNA) molecule.

"Expression" - the process by which a polypeptide is produced from a structural gene.

"Cloning vehicle" - is a plasmid or phage DNA or other DNA sequence which is capable of carrying genetic information into a host cell. A cloning vehicle is often characterized by one or more endonuclease recognition sites at which such DNA sequences may be cut in a determinable fashion without loss of an essential biological function of the vehicle. A cloning vehicle is a DNA sequence into which a desired DNA may be spliced in order to bring about its cloning into the host cell.

"Vector" - is a term also used to refer to a cloning vehicle.

"Plasmid" - is a cloning vehicle generally comprising a circular DNA molecule that is maintained and replicates autonomously in at least one host cell.

"Expression vehicle" - a vehicle or vector similar to a cloning vehicle but which supports expression of a gene that has been cloned into it, after transformation of a host. The cloned gene is usually placed under the control of (i.e. is operably linked to) certain control sequences such as promoter sequences.

"Host" - a cell that is utilized as the recipient and carrier of recombinant material.

"Homologous" - refers to a nucleic acid molecule that originates from the same genus or species as the host.

"Heterologous" - refers to a nucleic acid molecule that originates from a different genus or species than that of the host.

"Glycoprotein" - refers to a peptide molecule that has undergone glycosylation.

"Glycosylation" - refers to the addition of carbohydrate groups to a amino acid residues of a peptide molecule.

In recent years, transgenic animals have been developed for many purposes (Pinkert *et al.* 1990) (Wall *et al.* 1997). One premise, therefore, for the present invention is that by providing a transgenic animal capable of expressing phytase, the problems discussed above would be obviated. The options for heterologous phytase expression in animals include (i) salivary gland secretion of a phytase, (ii) pancreatic secretion of the enzyme into the small intestine along with the digestive enzymes, or (iii) secretion from the intestinal epithelial cells much like that of indigenous alkaline phosphatase and glycosidases (Low, 1989). The *E. coli* phytase would appear to be best suited for hydrolytic activity in the monogastric stomach

because the enzyme has a pH optimum in the range of 2.5 to 4.5 and it is resistant to pepsin, the predominant protease active in the stomach. The phytase has a periplasmic location in *E. coli* and has an N-terminal signal peptide sequence (Golovan et al., 1999) that seemed optimally adapted for secretion from the parotid gland. Phytase could be expressed in either the pancreas for secretion into the small intestine or it could be expressed in the intestinal epithelial tissue and secreted into the intestinal milieu. However, if these choices of expression locations were chosen, it would be necessary to select an enzyme active at the more neutral pH of the small intestine and one which was more resistant to pancreatic enzymes including trypsin, chymotrypsin and elastase.

Factors of importance in terms of the expressed enzyme when selecting a phytase for expression in the gastrointestinal tract include a pH that is optimum for activity, high catalytic activity, broad substrate specificity, and protease resistance. If any of these properties, or indeed others, is not acceptable, there are now sophisticated molecular methods for modifying the properties of an enzyme. These include site directed mutagenesis, random mutagenesis and various modifications of DNA shuffling (Harayama, 1998; Crameri et al., 1998).

Synthesis of phytase in the salivary gland and secretion in the saliva would, therefore, provide for early contact of the enzyme with phytic acid present in the feed and provide sufficient time for hydrolysis.

The salivary gland system of the pig consists of three pairs of glands, the parotid gland, which secretes through a duct on each cheek, and mandibular and submaxillary glands that have joint ducts that secrete beneath the front on the tongue. Saliva secreted in the pig via these ducts is discontinuous and is produced during consumption of solid foods, and can equal the weight of food consumed when water is limited during feed consumption (Corring, 1980; Arkhipovets, 1956). For example, the quantity of saliva produced by a 45 kg pig can vary from near zero when the pig receives a mainly liquid diet to 500 g when a dry diet is consumed without access to water. The salivary glands of the pig secrete amylase (Rozhkov and Galimov, 1990) and a variety of other salivary proteins and mucopolysaccharides.

To our knowledge no porcine genes coding for salivary proteins have been cloned. However, genes coding for major proteins secreted by the rat and mouse have been cloned and characterized. A multigene family encoding a group of unique proteins high in proline, the so-called proline-rich proteins (PRPs) are produced when either mice or rats consume tannins or are injected with isoproterenol.

It would be advantageous to develop an animal that is transformed to express phytase, preferably in the salivary gland. In such case, the phytate naturally occurring in the animal feed can be utilized by the animal without any additives being used. This will decrease the cost of animal production, and furthermore, will avoid polluting the environment with phosphorus. Therefore, the present invention aims to overcome the deficiencies of the prior art relating to increasing phytate utilization and, particularly, to provide transgenic animals which express phytase.

In the production of heterologous proteins by means of recombinant methods, several hurdles have been faced. One such hurdle that is often faced is the lack of required post-translational modification of the expressed protein thereby resulting in a protein that is structurally and/or functionally, different from the desired molecule. Glycosylation is one such post- translational modification that is desired. However, such modification is generally found to occur in more complex mammalian systems. Therefore in one embodiment of the present invention there is provided a method of producing recombinant glycoproteins.

In one embodiment, the present invention provides an animal capable of inducible or constitutive salivary expression of a heterologous protein. To illustrate this, the mouse was chosen as the animal model and the gene constructs used for transformation were created using the rat proline-rich protein (PRP) promoter/enhancer (inducible promoter) and the mouse parotid secretory protein (PSP) promoter/enhancer (constitutive promoter). In this illustration, phytase was used for expression in saliva.

After finding that an inducible phytase could be expressed in the parotid gland of mice the expression of the phytase transgene under the control of the constitutive PSP promoter was then tested. Two mice transgenic for the PSP construct were produced under contract at the University of Alabama.

Following the testing of the mice described above, transgenic pigs were developed by introduction into the genome a phytase transgene consisting of a constitutive promoter driving the synthesis of a highly active phytase. The pigs so generated were found to excrete less phosphorus in their feces than non-transgenic pigs.

Expression in the Salivary Glands

Saliva is a clear colorless fluid secreted by major salivary glands (parotid, submandibular, sublingual and minor salivary) that lubricates and cleans the oral structure, as well as initiates the process of digestion. The parotid glands are two of six major glands associated with the production of saliva. The parotid gland is composed mainly of two cell

types: acinar and interglobular duct cells. The acinar cells, which represent 75 to 85% of the tissue, are the sites of secretory protein synthesis (Frandson and Spurgeon 1992). Two very abundant proteins are produced by these cells: α-amylase (AMY-1) (2% of polyA RNA) (Madsen and Hjorth 1985), and parotid secretory protein (PSP) (10% of polyA RNA) (Shaw and Schibler 1986). Several constructs are now available which allow tissue-specific expression of a transgene in the salivary glands of mice.

The salivary secretion in pigs has not received the attention given to that of mice and humans. It was suggested that salivary secretion is discontinuous (less secreted between periods of meal consumption). Up to 500 g of saliva may be secreted by a 45 kg pig upon consumption of 500 g of dry feed (Corring 1980). Wide variations were detected in both the flow rate and electrolytes in saliva between animals and even between samples taken from the same animal on separate days (Tryon and Bibby 1966). Very little is known about the composition of pig's saliva or salivary enzymes. Salivary amylase was detected, although the quantity was 250 000 times less than that of pancreatic amylase, and 100 times less than in human saliva (Low 1989). There are no constructs known which would allow salivary gland-specific expression of transgene in pigs.

I) APPA Gene Under Control Of An Inducible Promoter

1) Construction of R15/APPA constructs (Inducible Promoter)

In this process, a plasmid is constructed by linking a promoter/enhancer for a saliva protein with the *APPA* gene, which codes for the bifunctional phytase, acid phosphatase. The *APPA* gene used in this construction was cloned from *E. coli* ATCC 33965 into pBR322. This is described above (Golovan et al., 2000).

Proteins, unusually high in proline, the so-called proline-rich proteins (PRPs), comprise about 70% of the total proteins in human saliva (Bennick 1982). Unlike the constitutive expression of the PRPs in humans, the salivary glands of mice, rats and hamster normally either do not express PRPs or express them in low levels. In the rat and mouse, PRP gene expression can be dramatically induced by diets high in tannins or by injection with the β-agonist isoproterenol (Carlson 1993). After 6 to 10 days of daily isoproterenol injection the PRPs comprised about 70% of the total soluble protein in parotid gland extracts. PRP cDNA and PRP genes have been cloned and characterized from rats (Clements *et al.*

1985), mice (Ann and Carlson 1985), hamsters (Mehansho *et al.* 1987), and humans (Kim and Maeda 1986).

Transgenic mice were used to locate the cis-acting DNA elements that are essential for salivary-specific and inducible expression of the rat proline-rich protein gene, R15. It was found that a parotid control region (-6 to -1.7 kb) upstream of the R15 promoter is capable of directing parotid-specific and isoproterenol-inducible expression of a heterologous promoter construct (Tu *et al.* 1993). The distal -10 to –6 kb region was shown to function as an enhancer, which can increase levels of expression more than 30-fold. The -6 to -1.7 kb region also seems to function as a locus control region (LCR), because it conferred copy number-dependent and chromosomal position-independent expression of a reporter gene in 15 out of 15 independent transgenic mice (Tu, Lazowski, Ehlenfeldt, Wu, Lin, Kousvelari, and Ann 1993).

We obtained the R15-PRP promoter from Dr. D.K. Ann as a plasmid -10*R15/*CAT, which placed the chloramphenicol acetyltransferase gene (CAT) under control of the inducible R15-PRP promoter. We decided to use the plasmid as a basis for transgene construction (Figure 1). Due to the absence of complete sequence information about the R15-PRP promoter (only 2 kbp out of 10 kbp was sequenced) we removed the R15-PRP promoter by Xho I digestion (Figure 1, step 1). Re-ligated plasmid was used as a template for PCR with CAT-ATG and CAT-TAA synthetic primers. The 4.3 kbp CAT_{PCR} fragment had the initiation site of the CAT gene substituted with the optimal eukaryotic initiation sequence (Kozak 1987). The fragment was purified by agarose gel electrophoresis, re-ligated to itself and used to transform *E. coli* (Figure 1, step 2). The CAT_{PCR} plasmid was digested with Nco I and filled-in using T4 DNA polymerase to generate a blunt end. After that, the CAT_{PCR} fragment was digested with Eco47III and purified by agarose gel electrophoresis (Figure 1, step 3). Three rare codons in the *APPA* gene were modified during the sub-cloning steps leading to the construction of the transgene. Specifically, the Ala_3 coding sequence was changed from GCG to GCC, the Pro_{428} sequence was changed from CCG to CCC, and the Ala_{429} sequence was changed from GCG to GCT. This modification was made in order to increase the possibility of transcription of the gene in eukaryotic cells. The APPA gene was amplified by PCR using the previously cloned *APPA* gene from the pBR322/*APPA* plasmid with the synthetic primers APPA-DRA and APPA-SMA. The 1.3 kbp $APPA_{PCR}$ fragment generated by PCR was digested with Dra I and Sma I and gel-purified (Figure 1, step 4). $APPA_{PCR}$ and CAT_{PCR} fragments were blunt end ligated to produce CAT/APPA+intron

vector (Figure 1, step 5), which was introduced into a DH5α strain of *E. coli*. The insert orientation was checked by restriction digest with Sal I and EcoR I. The transgene in CAT/APPA+intron was checked by sequencing both strands. To remove the SV40 small t intron the 2.3 kbp *APPA*/intron/polyA fragment was excised from a plasmid by Xho I and EcoR I digestion (Figure 1, step 6a), gel purified and digested by Dra I (Figure 1, step 6b). The 1.5 kbp (*APPA*) and 0.2 kbp (polyA) fragments were gel-purified and linked together in three way ligation with CAT_{PCR} digested with Xho I and EcoR I (Figure 1, step 6c). The resulting plasmids CAT/APPA and CAT/APPA+intron were digested with Xho I, gel-purified and re-ligated with R15-PRP promoter digested with Xho I (Figure 1, step 7). Because of the low efficiency of ligation the whole ligation mixture was used to transform *E.coli*, total plasmid DNA was prepared and run on the agarose gel. Plasmids which were larger than the original CAT/APPA (5.6 kbp) were eluted and re-transformed in *E.coli*. Plasmids with the R15-PRP insert (15 kbp) were identified by electrophoresing DNA from a single colony on an agarose gel. The correct orientation was identified by PCR with R15-UP1and APPA-DOWN2 synthetic primers. The plasmids R15/APPA and R15/APPA+intron were both digested with Hind III and Kpn I; transgenes were gel-purified and further purified using a Qiagen column (Figure 1, step 8).

Figure 18 illustrates the nucleic acid sequence for the plasmid containing the known segment of the R15/APPA + intron sequence including the vector sequences of pBLCAT3. The sequence of this plasmid is designated as SEQ ID NO:2.

Figure 19 illustrates the nucleic acid sequence for the transgene construct containing the known segment of the R15/APPA + intron sequence used for the generation of transgenic mice. The sequence of this transgene is designated as SEQ ID NO:3.

Figure 20 illustrates the nucleic acid sequence for the plasmid containing the known segment of the R15/APPA sequence including the vector sequences of pBLCAT3. The sequence for this plasmid is designated as SEQ ID NO:4.

The pBLCAT3 sequence indicated above is present in the CAT/APPA of Figure 1 and in the CAT/APPA+intron of Figure 2. This sequence was part of the original -10R15/CAT and a portion of it was carried through in the construction process.

Figure 21 illustrates the nucleic acid sequence for the transgene construct containing the known segment of the R15/APPA sequence used for the generation of transgenic mice. The sequence of this transgene is designated as SEQ ID NO:5.

2) Expression of SV40/APPA+intron in Cell Culture

To produce an SV40/APPA plasmid for expression of *APPA* in cell culture, the SV40 promoter/enhancer was amplified by PCR from the pSV-β-galactosidase plasmid (Promega) using the synthetic primers SV-HIND and SV-XHO. The SV40 promoter/enhancer fragment was digested with Xho I and Hind III, gel purified, and ligated into CAT/APPA digested with Xho I and Hind III (Figure 2).

Figure 22 illustrates nucleic acid sequence for the SV40/APPA + intron. The sequence for this plasmid is designated as SEQ ID NO:6.

We obtained a rat parotid acinar cell line PARC 5.8 (Quissell *et al.* 1998) that we intended to use for transient expression of the phytase transgene. The purpose was to test the efficiency of different constructs for transgene expression and also to detect any deleterious effects of phytase expression before introduction into the animals. We tried transient expression of the *APPA* gene using R15/APPA and R15/APPA+intron constructs but because of low transfection efficiency and/or low expression levels, we were unable to detect either phytase or β-galactosidase that we used as a control for transfection.

We exchanged the R15-PRP inducible promoter from the R15/APPA construct with the SV40 constitutive promoter-enhancer, which enables high level transient expression in different cell cultures. CHO, COS7 and HELA cell lines were screened for transient expression of the *APPA* phytase using the SV40 promoter/enhancer. All cell lines were maintained on DMEM/F12 (Sigma) cell medium with 10 % (wt/vol) heat-inactivated fetal bovine serum at 37°C in 5% CO_2 and 95% air. Cells were grown to 70 % confluence before transfection. Two hours before transfection the medium was exchanged with fresh medium. Cells were transformed with 5 μg of DNA per 60 mm culture plate (1:1 SV40/*APPA* and SV40/β-galactosidase) using the DNA-Calcium-Phosphate method of transfection (Gorman *et al.* 1983). After 6 hours of incubation the medium was removed and cells were subjected to glycerol shock for 3 min (Ausbel *et al.* 1992). Cells were washed with phosphate-buffered saline (PBS) and incubated in fresh medium under standard growth conditions. After 48 hours of incubation cell-free culture fluid was collected, the cells washed two times with PBS and lysed with 1ml of 1% (vol/vol) NP-40, 1mM disodium EDTA in Hanks balanced salts (HBSS) for 1 hour at 4°C. The phytase assay was performed in a final volume of 100 μl of 0.1 M sodium acetate/acetic acid buffer (pH 4.5) using sodium phytate (4 mM) as a substrate at 37°C. After 6 hours of incubation the reaction was stopped with 67 μl ammonium molybdate/ammonium-vanadate/nitric acid mixture and the concentration of liberated

inorganic phosphate determined at 405 nm (Engelen *et al.* 1994). One unit (U) of enzyme activity was the amount of the enzyme releasing 1 µmol inorganic phosphate per minute. The assay was performed in triplicate. As a control for endogenous phytase activity, non-transfected cell lines were used.

We did not detect endogenous phytase activity in non-transfected cell lines. Phytase activity was detected in all transfected cell lines, with COS7 cells expressing a total of 0.35 U of phytase in cell-free culture fluid (4 ml) and 0.0034 U in the cell fraction (1.1 ml) obtained from the same plate. The phytase activity produced by COS7 cells was 7 times higher than that of CHO and 35 times more than the HELA cell line. More than 99% of activity was located in cell-free culture fluid, which suggests that the expressed enzyme was exported out of the cell using the bacterial signal sequence. We were unable to detect expression of cytoplasmic β-galactosidase, which we wanted to use as a control for transfection efficiency.

3) Expression of R15-PRP/APPA in Transgenic Mice

Transgenic mice were generated using the constructs R15/APPA and R15/APPA+intron by Dr. C.A. Pinkert at the NICHD Transgenic Mouse Development Facility (NTMDF), University of Alabama at Birmingham, Alabama. The procedures followed in generating the mice have been standardized by the NTMDF and further information concerning this can be obtained at: http://transgenics.bhs.uab.edu/page1.htm, the content of which is incorporated herein by reference. This procedure involved the microinjection technique for transfecting mice with the desired nucleic acid sequence. To summarize, the sequences are microinjected into mouse zygotes and the surviving eggs are implanted into pseudopregnant recipient mice. The recipient mice then give birth to the resulting founder transgenic mice. It will be appreciated that various other methods of generating transgenic mice may be used in the present invention.

The R15/APPA transgene in mice was detected by PCR using the primers CAT-UP1 and APPA-DOWN2 that gives rise to a 700 bp fragment using the standard PCR conditions, except that the hybridization step was set at 51°C for 40 seconds and the polymerization step was at 72°C for one minute.

For the R15/APPA construct 8 PCR positive founder mice were obtained of which 4 were males and 4 were females. Three of the founders did not pass the transgene to progeny and were probably mosaics. For R15/APPA+intron 5 PCR positive founder mice were obtained, 3 were males and 2 were females, and one of them was found to be mosaic. At 10

to 12 weeks of age PRP production in the PCR positive progeny from different lines was induced for 10 days by daily intraperitoneal (ip) injection of 1mg isoproterenol dissolved in 100 µl sterile saline. To serve as a control several PCR negative progeny were also induced. No significant differences in weight were noticed between PCR positive and PCR negative progeny at either the beginning or end of the induction period. Saliva was collected before induction and at the end of the 10 day induction period.

To collect saliva, mice were lightly anesthetized with a ketamine/xylazine mixture (ip injection of 50 mg ketamine and 5 mg xylazine per kg body weight diluted in water) and saliva flow was induced by injection with pilocarpine/isoproterenol (ip injection of 0.5 mg pilocarpine and 2 mg isoproterenol per kg body weight dissolved in saline) (Hu *et al.* 1992). Between 100-250 µl of saliva was collected from each mouse over a 30 min period beginning 5 min after the pilocarpine/isoproterenol injection.

The saliva was collected from each mouse by holding it in one hand and withdrawing saliva from the corner of the mouth with a 20 µl pipetter. Collected saliva was transferred to a cold Eppendorf microcentrifuge tube containing 2 µl of 0.5 M EDTA (pH 8.0) and 4 µl of 10 mg/ml protease inhibitor Pefabloc (Boehringer Mannheim) dissolved in water. The tubes with saliva were kept on ice until assays were conducted. Phytase activity in the saliva was assayed as described for the SV40/APPA expressed in cell culture.

Phytase expression was not detected in either un-induced or in induced PCR negative mice. For PCR positive mice, phytase expression was not detected in those that were un-induced. However, phytase expression was observed for PCR positive mice that were induced. The results of this study are summarized in Table 1.

Even though it was possible to distinguish saliva from induced PCR positive from that of PCR negative mice in a phytase assay by a characteristic yellow color, saliva from some of the negative mice, when assayed, produced cloudiness that was impossible to remove by centrifugation and that affected spectrophotometer readings. We did not notice any gender differences in expression, both males and females were found to produce phytase in saliva. In three lines (all R15/APPA+intron) no phytase expression or very low level of expression (0.03-0.95 U/ml) was detected, in 4 lines the level of expression ranged from 7 to 87 U/ml, and two lines (both R15/APPA) produced very high levels of phytase in saliva, 252 and 547 U/ml.

These experiments demonstrated that phytase can be expressed at a very high level in the salivary glands of mice, without detrimental effects on the animals. We also were able to

produce progeny with an inducible salivary phytase from animals expressing the inducible phytase thereby documenting inheritance of the trait, and showing that the reproductive capability of animals was not affected. When the F2 generation of mice were tested for salivary phytase the level of phytase production was preserved.

Founders containing the transgene without the intron gave offspring that produced significantly higher levels of phytase. The SV40 intron in the R15/APPA+intron construct seems to cause a lower level of expression, and in three lines (A1f, A20f and B0m) the level of phytase was barely detectable. The level of phytase expression in A2m line (R15/APPA+intron) was 6.2 times lower than that of the B0m-intron line (R15/APPA).

Preliminary experiments showed that when the enzyme was analyzed by PAGE its size was increased from 42 kDa to 60 kDa. It is likely modified by glycosylation, but stable and active.

II) APPA Gene Under Control Of A Constitutive Promoter

1) Construction of the Lama2/APPA Transgene (Constitutive Promoter)

The murine parotid secretory protein (PSP) is the most abundantly expressed protein in the parotid gland of mice (Madsen and Hjorth 1985). After an hour of pulse labeling, the mouse parotid gland incorporates 65 to 85% of ^{14}C-leucine into this single protein (Owerbach and Hjorth 1980). It was estimated that PSP mRNA accumulates up to 50,000 molecules per cell and that from 3 to 5 molecules of PSP are produced for every molecule of amylase (Madsen and Hjorth 1985). Despite the predominance of the PSP in saliva its function is not well characterized.

The single-copy gene coding for PSP has been cloned and characterized. It has two alleles PSP^a (Shaw and Schibler 1986) and PSP^b (Owerbach and Hjorth 1980). The PSP^b allele is also expressed in the sublingual gland, but at 1/10 of the level found in the parotid gland. It was shown that 4.6 kbp of 5' flanking sequence of PSP^b is sufficient for salivary gland specific expression. The level of sublingual expression approached 100% of the PSP mRNA level, whereas the parotid expression did not exceed 1% (Mikkelsen *et al.* 1992), which demonstrates that regulatory sequences for sublingual and parotid expression are not identical. The level of expression was also dependent on the site of integration. The same construct was used for expression of the C-terminal chain of the human blood coagulation factor VIII, FVIII. A high level of FVIII mRNA was detected in the sublingual gland and a low level in the parotid gland. The transgenic lines also secreted the FVIII light chain into

saliva at the level of about 10 units per salivation (about 0. 05 ml of saliva) (Mikkelsen et al.,1992). Later the same group achieved a high level of parotid-specific expression that was similar or even exceeded that of the endogenous gene by using 11.4 kbp of 5' flanking sequences and 2.5 kbp of 3'flanking sequences (Larsen *et al.* 1994). The expression also seems to be position-independent and copy-number-dependent that could indicate the presence of a LCR in these sequences.

Lama 2 is a portion of the PSP gene and comprises an 18 kbp construct that is expressed in transgenic mice at up to 56% of the endogenous PSP gene.

Because a large part of Lama 2 had not been sequenced, the construct was first disassembled and subcloned into pBluescript KS(+) and after incorporation of the *APPA* gene, the Lama 2 was reassembled back (Figure 3). We used unique enzymes RsrII and SmaI to remove a 3.4 kbp fragment from Lama2, which was subcloned into the multiple cloning site (MCS) of pBluescript II KS(+) that was previously digested with KpnI and SmaI, using a KpnI-RsrII adapter (Figure 3, step 1).

KpnI* RsrII

TGGGAGGTCG

CATGACCCTCCAGCCAG

That allowed us to preserve the RsrII (CG/GWCCG) site and destroy the KpnI site (GGTAC/C> GGTAC/T), which would otherwise interfere with future cloning. The pKS/Lama construct was digested with ApaI and KpnI and used in a three-way ligation with the modified APPA (Figure 3, step 2). We designed two PSP/APPA constructs. One construct APPA-signal/APPA (Figure 3, steps 3a-7a) had the original bacterial signal sequence from the APPA protein having the following amino acid sequence:

Met-Lys-Ala-Ile-Leu-Ile-Pro-Phe-Leu-Ser-Leu-Leu-Ile-Pro-Leu-Thr-Pro-Gln-Ser-Ala-Phe-Ala

We also modified a sequence near the ATG codon to resemble the optimal mammalian Kozak sequence (GCC GCC A/GCC ATG G) (Kozak 1987), but we did not mutagenize the +4 position because it would change Lys to Glu in the signal sequence with possible deleterious consequences for protein export. This optimized sequence was used in our previous construct R15/APPA and led to high levels of phytase production. We checked the APPA bacterial signal sequence using the PSORT computer neural network trained on eukaryotic signal sequences and further described at http://psort.nibb.ac.jp:8800/ (Nakai and

Kanehisa 1992). The APPA bacterial signal sequence was recognized as an efficient leader peptide and the cleavage site was correctly predicted. PSORT also predicted that there is a high probability that phytase would be exported correctly outside of the cell. There were also publications showing that some bacterial signal sequences might function efficiently in mammalian cells (Williamson *et al.* 1994) (Hall *et al.* 1990). Our experiments using cell culture demonstrated that the APPA signal was correctly processed with export of phytase outside of the cell.

Experiments using cell culture cannot predict the direction of export and if phytase were exported into blood vessels instead of salivary ducts that could lead to deleterious effects. That is why we also designed a second construct PSP-signal/APPA (Figure 3, steps 3b-7b) that would preserve the original PSP signal amino acid sequence:

Met-Phe-Gln-Leu-Gly-Ser-Leu-Val-Val-Leu-Cys-Gly-Leu-Leu-Ile-Gly-Asn-Ser-Glu-Ser

This leader peptide was also efficiently recognized by PSORT with the correct cleavage site (Nakai and Kanehisa 1992). In this construct we also preserved the original PSP sequences near the ATG start codons, which may not be optimal, but could be important in regulation of gene expression. The APPA gene for both constructs was amplified by PCR using as the template our previous transgenic construct R15/APPA that possessed the optimal Kozak sequence and the modified codons for residues Ala3, Pro428 and Ala429 as described earlier. For the APPA signal/APPA construct two synthetic primers were used which introduced a Cla1 site near the ATG codon (APPA-CLA) and a Kpn1 site near the TAA stop codon (APPA-KPN). The $APPA_{PCR}1$ product was digested with Cla1 and Kpn1. The Cla1 site was also introduced into Lama 2 using pKS/Lama 2 as template for PCR. LAMA-UP primer was located upstream of Apa1 site and the LAMA-CLA primer introduced the Cla1 site near ATG codon (Figure 3, step 3a). $Lama_{PCR}1$ product was digested with Cla1 and Apa1 (Figure 3, step 4a). pKS/Lama (Apa1-Kpn1), $Lama_{PCR}1$ (Apa1- Cla1) and $APPA_{PCR}1$ (Cla1-Kpn1) were combined together in a three-way ligation reaction (Figure 3, step 5a). The recovered pKS/Lama/APPA plasmid was digested with RsrII, Sma1 and inserted back into Lama2 (Figure 3, step 6a).

For the PSPsignal/ APPA construct, the synthetic APPA -KPN primer was used with the synthetic APPA -MATURE primer, which produced phytase without a signal sequence. The $APPA_{PCR}2$ product was blunt-ended using T4 DNA polymerase and digested with Kpn1. The PSP signal sequence was produced using the LAMA-UP and LAMA -SIGNAL primer

(Figure 3, step 3b). The Lama$_{PCR}$2 was blunt-ended using T4 DNA polymerase and digested with ApaI (Figure 3, step 4b). pKS/Lama (ApaI-KpnI), Lama$_{PCR}$2 (ApaI-blunt) and APPA$_{PCR}$2 (blunt-KpnI) were combined together in a three-way ligation reaction (Figure 3, step 5b). The recovered pKS/Lama/APPA plasmid was digested with RsrII, SmaI and inserted back into Lama2 (Figure 3, step 6b).

Even though both constructs were successfully produced we decided to use Lama2/APPAsignal/APPA for the generation of transgenic mice, because we have results from our previous transgenic constructs R15/APPA and R15/APPA+intron which demonstrated that phytase with optimized Kozak sequence and the APPA signal peptide was synthesized at a high level in salivary glands after induction and was efficiently exported into the salivary duct. The Lama2/APPA vector was digested with XhoI and NotI, and the transgene was gel-purified and further purified using a Qiagen column (Figure 3, step 7a).

2) Sequence of the Lama2/APPA Construct

A large segment of the Lama2 construct (Laursen and Hjorth 1997) used for construction of the Lama2-APPA transgene had not been reported in GenBank prior to our research. To ensure that we could more clearly describe the transgene construct, and furthermore to avoid the introduction of deleterious DNA sequences from the mouse into the pig in the process of generating transgenic pigs, we sequenced the Lama2-APPA plasmid on both strands. Figure 4 illustrates schematically the structure of the Lama2-APPA plasmid. Figure 5 illustrates the nucleic acid sequence (SEQ ID NO:1) of such plasmid. The full transgene sequence was reconstructed from overlapping DNA sequences using the Contig Assembly Program (CAP) (http://hercules.tigem.it/ASSEMBLY/assemble.html) developed by Huang (1996; 1999) and then inspected manually for sequencing errors. The transgene sequence was checked for the presence of interspersed repetitive elements using the computer program RepeatMasker (Smith and Green, RepeatMasker at http://ftp.genome.washington.edu/cgi-bin/RepeatMasker). It was found that 26 % of the transgene sequence was composed of repetitive elements (Table 2). However, such repetitive elements are widely present in all mammalian genomes. For example, up to 50% of the human genome is derived from repetitive elements (Smit 1996; Kazazian 1998).

Figure 23 illustrates the nucleic acid sequence (SEQ ID NO:7) of the Lama2/APPA transgene construct.

The Lama2 high level expression cassette (Laursen and Hjorth 1997) contains the enhancer region and the promoter of the *Psp* gene in the parotid gland. High expression was

shown to be dependent on regulatory elements between –11.5 kb and –6.5 kb and/or between +8.3 kb and +10.9 kb. Svendsen et al. (1998a) showed that a 1.5 kb sequence between –3.1 kb and –4.6 kb had properties of a parotid and sublingual specific enhancer and was designated as the PSP proximal enhancer. Furthermore, they showed that transgenes containing the PSP promoter and 5' flanking region located between –3.6 kb and –4.3 kb contained sequence information necessary to direct salivary gland specific expression.

Screening the transgene with RepeatMasker did not reveal the presence of any full-length active autonomous elements. The repeats present were extensively modified by insertions and deletions. The *blastx* program was also used to compare the transgene sequence translated in all reading frames against the National Center for Biotechnology Information (NCBI) protein sequence database (http://www.ncbi.nlm.nih.gov/BLAST/) (Altschul *et al.* 1990;Gish and States 1993;Terada and Nakanuma 1993). A region of DNA from 861 to 2180 was found that might code for parts of a protein with limited homology (38-58% identities) to the C-terminus of several human and mouse reverse transcriptases. However, the region was extensively modified by mutations with multiple frame shifts and inversions, and probably represented remnants left from the reverse transcriptase gene of a LINE element. It is unlikely that it would be active, due to extensive modifications in the amino acid sequence such that only 18% of the full reverse transcriptase sequence was present and the highly conserved amino acid motif (Y/FXDD) was absent from the sequence (Xiong and Eickbush 1990). The complete sequence was also scanned for the presence of open reading frames (ORFs) that code for proteins using the program GENSCAN (http://CCR-081.mit.edu/GENSCAN.html) (Burge and Karlin 1997). Only one gene was found and it corresponded to the *APPA* phytase gene. GENSCAN unexpectedly predicted a different N-terminus for the phytase than would have been expected from the sequence. However, that could have resulted from the lower accuracy of GENSCAN for detecting initiation sites (Burge and Karlin 1998).

3) Generation of Transgenic Mice Expressing a Constitutive Salivary Phytase

In the following description, a pair of founder mice, incorporating the phytase gene and a constitutive promoter, were prepared under contract by the University of Alabama. As will be discussed, these founders were used to produce offspring, which were then analyzed for the presence of the phytase gene by PCR and animals containing the gene were then tested constitutive salivary phytase production.

Two transgenic founder mice (a black male and a white female, 3-1) containing the phytase transgene were received from the NICHD Transgenic Mouse Development Facility at the University of Alabama. The black male was negative for salivary phytase, but the female, 3-1, exhibited a salivary phytase activity of 30 U/ml. Progeny produced by crossing the black male with 4 CD-1 females produced 9 out of 25 females and 13 out of 26 males that were PCR positive. All progeny were negative for salivary phytase. The female founder, 3-1, was out-crossed with a CD-1 male to produce 3 litters for a total of 35 offspring. Of the progeny from these matings one phytase positive G1 male was obtained. When the G1 male was outcrossed with 6 CD-1 females, of the 6 litters 20/34 males were PCR positive and salivary phytase positive and 21/28 females were PCR positive and salivary phytase positive (Table 3). The salivary phytase activity of different offspring from the same first generation (G1) male ranged from 1.3 to 71.2 U/ml. There was no significant difference in the phytase activities between male or female mice.

PCR assays for identification of the transgenic mice were carried out with an initial heating step at 95°C for 3 min, 40 cycles using 95°C for 30 sec, 54°C for 30 sec and 72°C for 1 min) using the following primers: APPA-UP2 and APPA-KPN (Figure 6).

The phytase assays were conducted as described above for the R15-PRP/APPA phytase expressed in cell culture.

4) Production of Transgenic Pigs Containing the Phytase Transgene Lama 2/APPA

Transgenic pigs were produced using Yorkshire and Yorkshire/Landrace cross gilts as the embryo donors and Yorkshire sows as the recipients. The experimental procedure used was similar to that described by Wall et al. (1985). The detailed procedure is described below. The Lama2/APPA construct with the APPA signal peptide was used as the transgene for microinjection.

Methodology for the generation of transgenic pigs

The following is a description of the preferred method of generating transgenic pigs according to the invention. However, it will be apparent to those skilled in the art that various other methods are also applicable.

a) Superovulation of prepuberal gilts and sows.

Selected Yorkshire or Yorkshire/Landrace cross gilts between 70 to 80 kg were superovulated by intramuscular injection of 2000 IU of pregnant mare's serum gonadotropin

(PMSG, Ayerst Veterinary Laboratories), followed by 700 IU human chorionic gonadotropin (HCG, Ayerst Veterinary Laboratories) 60 to 72 hours later, administered in the same manner. The gilts were artificially inseminated three times with a 16 hour interval between inseminations using semen from a high breeding index Yorkshire boar. Twenty-four hours after the last insemination, the gilts were slaughtered and the reproductive tract recovered.

b) Synchronization of estrus in recipients

Estrus was synchronized in experienced recipient sows as described for donor sows. Since synchronization and not superovulation was the goal, hormone levels were reduced to 500 IU for PSMG and 500 IU for HCG. PMSG was given the day the sow's litter was weaned, followed in 72 hours by HCG and surgery for embryo transfer was performed 54 hours thereafter.

c) Embryo collection

Reproductive tracts were collected at the abattoir, inserted into bags, sealed and the bags immersed in water at 39°C for transport to the laboratory. Recovery of the embryos and microinjection with the transgene was conducted in a laboratory maintained at 32 to 33°C. The oviducts were dissected from the tracts and flushed, using a syringe and a feeding tube, with 15 ml of pre-warmed HBECM-3 medium (Dobrinsky *et al.* 1996). The media was collected in a 100 mm Petri dish and placed in an incubator at 38.5°C with an atmosphere of 5% (vol/vol) of CO_2, 5% (vol/vol) O_2 and the balance N_2. After all tracts were flushed, embryos were individually collected from the flushed media using a polished transfer pipette. Embryos were rinsed twice in 3 ml volumes of pre-incubated BECM-3 and placed in 100 µl of pre-incubated BECM-3 under 3 ml of filter sterilized mineral oil until injected.

d) Pronuclear injection

Embryos from one gilt were collected and placed in one ml of pre-warmed HBECM-3 in a 1.5 ml centrifuge tube and centrifuged for 6 min at 14,000 x g (Wall *et al.* 1985). The embryos were then collected and placed in an injection dish with 40 µl of pre-warmed HBECM-3 covered with 2.5 ml of filter sterilized mineral oil. The pronucleus in each embryo was injected (Gordon *et al.* 1980) with three picolitres of Lama2/APPA DNA in solution at a concentration of 5 ng of DNA per µl in 10 mM Tris, pH 7.5, 0.1 mM EDTA. After injection, the embryos were placed in dishes containing 100 µl of pre-incubated

BECM-3 under 3 ml of filter sterilized mineral oil. After all embryos were injected, which took no more than 4 hours since collection of reproductive tracts, the embryos were transferred to 1.8 ml cryotube (Nunc) containing 1 ml of pre-warmed HBECM-3 and transported in an incubator at 38.5°C to the swine surgery.

e) Embryo transfer

Recipient sows were anesthetized by intravenous injection of 500 mg Brietol and anesthesia maintained by inhalation of 3% halothane with 4 litres per min of nitrous oxide and 4 litres per min oxygen. The oviducts were exposed through a laparotomy, just off the dorsal midline, and a catheter, containing 20 to 35 injected embryos and 3 to 6 untreated embryos, was passed into the infundibulum and down the oviduct to the isthmus and emptied. The oviduct was returned to the abdominal cavity and the incision closed.

f) Growth of pigs

New-born piglets were kept together until weaning. At that time males and females were separated and penned with non-transgenic same sex pigs of a similar age from other litters. The pigs are fed *ad libitum* starter rations until 25 kg wt , grower diet from 25 to 60 kg wt and finisher diet from 60 kg to market weight. Water is available *ad libitum*. Transgenic pigs 167-02, 282-02 and 282-04 were maintained on a low phytate ration until 85, 50, and 50 days of age, respectively, and then switched to the grower ration. All other transgenic pigs were given the standard high phosphorus diets.

The diets were provided as pelleted formulations during the weanling, grower and finishing phases are shown in Tables 4 and 5. The vitamin and mineral mixes included in the diets are shown in Tables 6 and 7.

PCR analysis

Tail segments from newborn piglets were collected and slices of each placed in 600 µl of 50 mM NaOH and heating at for 95°C for 15 minutes. The suspension was neutralized with 50 µl of 1 M Tris (pH 8.0) and insoluble materials removed by centrifugation for 5 min in a microcentrifuge. A 2 µl sample of each was used for PCR with primers APPA-UP2 and APPA-KPN.

The primers produce a 750 bp fragment if the transgene is present. As a positive control PIG-BGF and PIG-BGR primers were used to detect the porcine β-globin gene from

the same DNA preparation (Heneine and Switzer 1996). The PCR reaction was performed using the same conditions as described for detection of the phytase transgene. As a negative control genomic DNA from a non-transgenic pig was used in the PCR reaction, for a positive control this DNA was spiked with a known amount of transgene (1 gene copy/per genome).

When a positive signal was identified by PCR for pig 167-02 (Figure 3) another DNA preparation was made and two more pairs of PCR primers were used to test for gene integrity (Figure 4) APPA-MATURE with APPA-KPN, and APPA-MATURE with APPA-DOWN2

PCR conditions were similar to those described previously.

Extraction of DNA from blood for PCR analysis

The method for extraction of DNA from blood was based on a method described by Higuchi (1989) with some modifications. A 100 µl volume of whole blood was mixed with 200 µl of lysis buffer (10 mM Tris-HCl, 0.32 M sucrose, 5 mM $MgCl_2$, 1% (vol/vol) Triton X-100, pH 7.5.), mixed briefly and incubated on ice for 5 min. The sample was then centrifuged at 14,000 x G for 3 min, and the supernate discarded. The sediment was suspended in lysis buffer, mixed, incubated and centrifuged. This procedure was repeated 2 more times, or until no hemoglobin remained. The sediment was dissociated in 100 µl of 50 mM NaOH, mixed and heated at 100°C for 10 min. The contents were cooled, 10 µl of 1 M Tris-HCl (pH 8.5) added and mixed briefly. The sample was then centrifuged at 14,000 x g for 2 min and 2 µl of the supernate used for analysis by PCR.

The PCR reaction mixture with a total volume of 40 µl consisted of; 23.8 µl of distilled water, 4 µl of 10 X Gibco BRL PCR buffer, 1.2 µl of 50 mM $MgCl_2$, 0.8 µl of 10 mM dNTPs, 40 pmol of each of the forward and reverse primers in 8 µl, 2 µl of template DNA and 0.2 µl of *Taq* DNA polymerase (Gibco BRL, 5 U/µl). The amplification procedure was performed with an initial heating step at 95°C for 3 min followed by 40 cycles of 95°C for 30 sec, 54°C for 30 sec and 72°C for 60 sec.

The transgenic pigs were detected with primers for the *APPA* gene (APPA-KPN with APPA-UP2), and as a control PIG-BGF with PIG-BGR primers were used for detection of the porcine β-globin gene.

Saliva collection from pigs for phytase assays and weighing of pigs

Weanling pigs were sampled for salivary phytase by wiping under the tongue with a cotton tipped applicator, breaking the stick off and centrifuging the applicator tip in a 0.4 ml

microcentrifuge tube, with a hole in the bottom, contained within a 1.5 ml microcentrifuge tube. Grower and finishing pigs were sampled using 1.5 inch long #2 dental cotton absorbent rolls (Ash Temple Sundries Ltd, Don Mills, ON) attached to dental floss. These were centrifuged in 1.5 ml microcentrifuge tubes with holes in the bottom while contained in larger tubes. The saliva was collected from the larger tube and stored at –20°C until analyzed.

Saliva was collected and pigs were weighed at weekly intervals.

Analysis for phytase activity.

Saliva samples were either assayed directly or after dilution in 0.1 M acetate buffer pH 4.5. Phytase was assayed in 200 µl of 0.1 M sodium acetate buffer (pH 4.5) using sodium phytate (4 mM) as a substrate at 37°C. After 10 min of incubation the reaction was stopped by addition of 133 µl ammonium molybdate/ammonium vanadate/nitric acid mixture and the concentration of liberated inorganic phosphate determined at 405 nm (Engelen, van der Heeft, Randsdorp, and Smit 1994). This and all other assays were performed in triplicate. One unit (U) of enzyme activity was the amount of the enzyme releasing 1 µmol of inorganic phosphate per minute.

Assays for salivary phytase and for phytase in blood samples were conducted as previously described for saliva samples. A reagent blank with blood added at the same concentration as the samples assayed was subtracted from the sample readings.

Collection of fecal materials and analysis for total phosphorus

Fresh feces were collected from each pig during the grower and finisher phases. Samples were placed in aluminum trays closed with a wax paper top and immediately frozen, and kept frozen until they were lyophilized for analysis. After lyophilization the samples were transferred to room conditions overnight to reach equilibrium in moisture content. The samples were separately ground with a mortar and pestle until homogenous and sealed in plastic containers until analyzed further. Dry matter content of samples was analyzed according to AOAC (Association of Official Analytical Chemists (AOAC) 1984) by heating 1 gram samples at 110°C for 4 hours and cooling in a desiccator prior to weighing. To analyze total phosphorus content, samples were heated at 550°C in a muffle furnace and 10 ml of 10 M HCl added and heated to boiling. The contents from each sample was quantitatively diluted to 250 ml with water and inorganic phosphorus content was measured by the method of Heinoen and Lahti (1981).

Purification of the *E. coli* produced phytase and pig salivary phytase

The APPA phytase was over expressed in *E. coli* strain BL21(DE3) and the EDTA lysozyme extract fraction purified on DEAE-Sepharose and Sephadex-G75 as described by Jia et al. (1998). The pig phytase was purified by chromatography on DEAE-Sepharose and the band of enzyme eluted with a sodium chloride gradient was further purified by Chromatofocusing using a pH gradient from pH 4.0 to 7.0.

SDS-PAGE analysis and Silver Staining

Sodium dodecylsulfate polyacrylamide gel electrophoresis was performed using a 10% gel as described by Laemmli (1970), except that protein in the sample buffer was heated at 70°C for 10 minutes. Samples were stained with silver as described by Nesterenko et al. (1994).

Preparation of a monoclonal antibody specific for the *APPA* encoded *E. coli* phytase

Monoclonal antibodies specific to the *E. coli APPA* encoded phytase were prepared according to the procedures of Galfrè and Milstein (1981). Briefly, two female Balb/c mice were immunized 7 times over a period of 59 days with a purified APPA enzyme preparation. Mouse spleens were harvested, and the cells therein fused with an NS-1 myeloma cell line (Kohler and Milstein, 1976). Fused cells were selected for their ability to grow in media containing hypoxanthine, aminopterin, and thymidine (HAT). Western blotting and Enzyme-Linked Immunosorbent Assays (ELISA) were used identify those clones capable of secreting an antibody into the culture medium that recognized epitopes on both the *E. coli* and pig derived APPA enzyme. Clones secreting a desirable antibody were subcloned twice to ensure a pure culture of antibody secreting hybridomas.

Production of Polyclonal Antibodies Against the Purified *E. coli* derived APPA Phytase

Antibodies were prepared in two New Zealand White Rabbits by two intramuscular injections at different sites in the thigh of 50 µg of purified *Escherichia coli* derived APPA phytase in 0.5 ml of a 1:1 mixture of phosphate-buffered saline (PBS) and Freund's Complete Adjuvant. This was followed by repeat injections of 20 µg each of phytase in a 1:1 mixture of PBS and Freund's Incomplete Adjuvant on days 4, 19, 25, and 39. Blood was collected via heart puncture on day 42. The serum was separated from the cell fraction and used as the

source of antibodies. The basic procedures for antibody production are described in Harlow and Lane (1988).

Western blotting

Western blotting was performed as described by Towbin et al. (Towbin *et al.* 1979).

Deglycosylation of pig phytase was done according to protocols, Roche Molecular Biochemicals, with following modifications. Protein in 50 mM Tris (pH 8.0), 1 mM EDTA, 1% SDS, 1% 2-mercaptoethanol was denaturated by heating at 95° C for 3 min. Than protein was precipitated with chloroform-methanol method (Wessel and Flugge 1984) and resuspended at 100 µg/mL in 20 mM Sodium Phosphate (pH 7.2) with 1% Triton X-100. Complete deglycosylation of 5 µg in 50 µL phytase was carried out overnight at 37°C using 1 unit (U) N-glycosidase F, 1.2 mU O- glycosidase and 1 mU neuraminidase (Boehringer Mannheim GmbH). After incubation 0.5 µg of protein was run on the SDS gel.

Staining of glycoproteins

This staining was done using DIG Glycan Detection Kit (Boehringer Mannheim) according to manufacture instructions (O'Shannessy *et al.* 1987).

Statistics on the generation of transgenic pigs

The statistics on embryos recovered, microinjected and transferred into donor sows is shown in Table 8. A total of 4147 embryos injected with the transgene and 675 untreated embryos were introduced into 140 recipient sows with an average of 30 injected embryos and 5 uninjected embryos. All offspring were tested for the presence of the transgene in tissue biopsy, in blood by PCR analysis, and by an assay for phytase activity in the saliva.

Table 9 lists the transgenic pigs that were produced, their birth dates, sex and salivary phytase levels. There were 31 pigs transgenic for the phytase gene out of 203 live piglets born from embryos microinjected. These were detected by the presence of the gene in blood samples using the standard primer set, APPA-UP2 and APPA -KPN, but only 14 were detected by analysis of tail DNA preparations using the standard primer set. When the negative samples were reanalyzed using the primer set LAMA-UP1 and APPA–down4 (Figure 8) a further 8 tail DNA samples were found to be positive. Purification of the tail biopsy DNA probably would have led to all being PCR positive for the phytase transgene.

Characteristics of the phytase transgene in transgenic pig 167-02

The application of PCR to detection of transgenic pigs is exemplified by analysis of litter 167 in which one of 7 piglets tested, including one that was stillborn and one that was crushed by the sow after birth, one live piglet designated 167-02 was identified as positive for the APPA gene by generation of a PCR product (Lane 2) of approximately 750 bps from the tail chromosomal DNA (Figure 7). No rearrangements of the APPA gene were detected as documented by the positive PCR results using primers directed to the 3' region (lane 2) the whole gene (lane 3) and the 5' region (lane 4) of the APPA gene (Figure 8).

Salivary phytase and weight gain during growth of transgenic and non-transgenic penmates.

Data on salivary phytase activity and weight gain are shown for five transgenic pigs and for weight gains of their non-transgenic penmates in Figures 9, 10, 11, 12 and 13. The phytase activity in the saliva varied substantially from one sampling time to the next. This variability was attributed to a combination of environmental factors including whether the animal had just consumed food or water, and regulation of parotid and saliva secretion in relation to food and water consumption. The weight gains during growth of the five transgenic pigs was within the range of the weight gains of the normal non-transgenic pigs.

With the exception of 167-02 the growth rate of the transgenic pigs was similar to that of the non-transgenic litter mates.

Phosphorus content in the fecal materials from transgenic and non-transgenic pigs.

The phosphorus content of fresh fecal samples from three of the transgenic founder pigs, 167-02, 282-02, 282-04, 405-02 and 421-06 receiving weaning, grower or finisher ration is shown in Table 9. The phosphorus content of the feces of the transgenic pigs ranged from 1.59 to 2.26% while that of the non-transgenic penmates ranged from 1.61 to 2.76 %. The reduction in fecal phosphorus ranged from a maximum of 26% to a minimum of 8%. In most cases the differences were at the 99% level of significance. The ages of the pigs at the time of fecal sampling and the corresponding phytase activities are shown in Figures 9, 10, 11, 12 & 13. The rations fed contained a supplement of readily available phosphorus suitable for maximizing growth of non-transgenic pigs. Since the reduction in fecal phosphorus is measured in transgenic pigs receiving a diet high in mineral phosphorus it is very likely that the fecal phosphorus would be substantially lower if the diet lacked mineral phosphorus. Under these conditions the phosphorus released from phytate would provide a substantial

proportion of the dietary phosphorus and little would reach the large intestine and be excreted in the feces.

Transmission of the phytase transgene (to be completed)

When semen from the transgenic boar 167-02 was used to inseminate four Yorkshire gilts all four sows had litters in which 4 out of 8, 2 out of 9, 7 out of 8 and 2 out of 5 of the piglets were transgenic for the phytase gene (Table 11). The PCR data for litter 154 that documents the presence of the transgene is shown in Figure 14. All pigs containing the gene exhibited phytase activity in the saliva, and it ranged from 341 to 10,077 units per ml. Half of the transgenic piglets had salivary phytase activities of greater than 2000 units per ml. The specific activity of the phytase in the saliva ranged from 39 U/mg protein to a high of 706 units/mg protein.

This data documents that the gene was transferred and that the level of phytase expression observed in the founder was preserved in the first generation of pigs. Both male and female pigs at 11 days of age exhibited high phytase activity.

Characteristics of the phytase enzyme synthesized in the salivary glands of the pig

The phytase enzyme was purified to homogeneity from *E. coli* and from saliva collected from transgenic pig 167-02. Silver stains of the purified enzymes after SDS-PAGE are shown in Figure. 15. The *E. coli* derived enzyme has a molecular mass of approximately 45 kDa while that produced by the pig was about 55 kDa. The enzymes were also electrophoresed as before, transferred to nitrocellulose and stained for glycoproteins. The second part of Figure 15 shows that the pig APPA protein is glycosylated. Figure 15B shows that treatment of the pig phytase with deglycosylation enzymes changes the size of the phytase from 60 kDa to 45 kDa, an observation that confirms the glycosylated nature of the recombinant phytase produced in the saliva of the pig.

The data in Figure 16 shows that the pig phytase is homologous with the *E. Coli* enzyme despite their difference in size.

The purified pig phytase had K_m and V_{max} values of 0.33 mM and 624 units per mg of protein, respectively. Golovan et al. (2000) previously reported the K_m and V_{max} for the *E. coli* enzyme to be 0.63 mM and 2325 units per mg of protein. Thus the salivary phytase exhibits approximately 25% of the activity of the *E. coli* enzyme. This reduction in activity may be due to glycosylation that either modifies the catalytic site of the enzyme or otherwise leads to the formation of an enzyme with lower catalytic activity.

The latter finding of the production of a glycosylated protein suggests a method of producing such proteins using transgenic animals. Currently, although recombinant methods are available for producing proteins in host cells, it is often found that the mature peptide lacks the glycosylation normally associated with proteins produced by higher life forms. Insulin is an example of such protein. The findings of this study suggest that one means of producing the desired glycoproteins would be to generate transgenic animals such as the pig, that have been transformed, by known methods or the method described above, with a gene encoding the desired protein. When expressed by such animal, the subject protein would be produced and would undergo post-translational processing in the cell including the step of glycosylation. Thus, the invention contemplates a general method of producing such glycosylated proteins. Further, the invention contemplates a method of producing glycosylated proteins through the expression in and isolation from the saliva of an animal that has been transformed with a gene encoding such protein, and wherein such gene is operably linked to a saliva protein promoter or enhancer.

Various methods are known in the art for the collection of glycoproteins from the parotid gland of the pig for various applications. For example, surgical techniques have been published by Denny et al. (1972) for the collection of secretions from the parotid gland and submandibular salivary ducts.

Test kit for detection of the APPA phytase protein in pigs

The monoclonal antibodies produced against the APPA phytase expressed in *E. coli* reacted with the APPA phytases produced in the saliva of transgenic mice and pigs (Figure 17). Immunological detection of phytase in saliva provides definitive proof that the phytase secreted in transgenic pig saliva is a product of the *APPA* gene expressed in the pig salivary gland. This serves as a reliable method to document phytase production in transgenic pigs.

A further test would also be obtainable using the polyclonal antibodies discussed above.

The DNA sequence encoding phytase may be obtained from a variety of sources such as microbial, plant or animal sources. Preferably, the DNA sequence is obtained from a microbial source such as bacteria. Most preferred DNA sequences are obtained from *Escherichia coli*.

The cloning of a gene or a cDNA encoding a phytase protein may be achieved using various methods. One method is by purification of the phytase protein, subsequent

determination of the N-terminal and several internal amino acid sequences and screening of a genomic or cDNA library of the organism producing the phytase using oligonucleotide probes based on the amino acid sequences. If at least a partial sequence of the gene is known, this information may be used to clone the corresponding cDNA using, for instance, the polymerase chain reaction (PCR) (PCR Technology: Principles and Applications for DNA Amplification, (1989) H. A. Ehrlich, ed., Stockton Press, New York; the contents of which are incorporated herein by reference). It will be evident to those skilled in the art that the cloned phytase gene described above may be used in heterologous hybridization experiments, directed to the isolation of phytase encoding genes from other microorganisms.

The DNAs encoding phytase or individual fragments or modified proteins thereof can be fused, in proper reading frame, with appropriate regulatory signals as described in detail below, to produce a genetic construct that is then amplified, for example, by preparation in a bacterial (e.g., *E. coli*) plasmid vector according to conventional methods. Such methods are described in, for example, Sambrook et al., Molecular Cloning: A Laboratory Manual (Cold Spring Harbor Press 1989), the contents of which are incorporated herein by reference. The amplified construct is thereafter excised from the vector and purified for use in producing transgenic animals.

The desired protein may also be produced as a fusion protein containing another protein. For example, the desired recombinant protein of this invention may be produced as part of a larger recombinant protein in order to stabilize the desired protein. Useful modifications within this context include, but are not limited to, those that alter post-translational modifications, size or active site, or that fuse the protein or portions thereof to another protein. Such modifications can be introduced into the protein by techniques well known in this art, such as by synthesizing modified genes by ligation of overlapping oligonucleotides or introducing mutations into the cloned genes by, for example, oligonucleotide-mediated mutagenesis.

The cloned phytase gene may be used as starting materials for the construction of improved phytases. Improved phytases are phytases, altered by mutagenesis techniques (e.g. site-directed mutagenesis, or directed evolution), which have properties that differ from those of wild-type phytases (Kuchner and Arnold 1997). For example, the temperature or pH optimum, specific activity, temperature or protease resistance may be altered so as to be better suited for a particular application.

A choice of expression in cellular compartments (such as cytosol, endoplasmic reticulum) or extracellular expression can be used in the present invention, depending on the

biophysical and biochemical properties of the phytase. Such properties include, but are not limited to pH sensitivity, sensitivity to proteases, and sensitivity to the ionic strength of the preferred compartment. The DNA sequence encoding the enzyme of interest should be modified in such a way that the enzyme can exert its action at the desired location in the cell. To achieve extracellular expression of the phytase, the expression construct of the present invention utilizes a bacterial signal sequence. Although signal sequences that are homologous (native) to the animal host species are preferred, heterologous signal sequences, i.e. those originating from other animal species or of microbial origin, may be used as well. Such signal sequences are known to those skilled in the art.

All parts of the relevant DNA constructs (promoters, regulatory, secretory, stabilizing, targeting, or termination sequences) of the present invention may be modified, if desired, to affect their control characteristics using methods known to those skilled in the art. The cis-acting regulatory regions useful in the invention include the promoter that drives expression of the phytase gene. Highly preferred are promoters that are specifically active in salivary gland cells. Among such promoters, highly preferred are mouse parotid secretory protein (PSP) promoter, rat proline-rich protein (PRP) promoter, human salivary amylase promoter, mouse mammary tumor virus promoter (Samuelson 1996). Among the useful sequences that regulate transcription, in addition to the promoters discussed above, are enhancers, splice signals, transcription termination signals, and polyadenylation sites. Particularly useful in this regard are those that increase the efficiency of the transcription of the genes for phytase in the salivary gland or other cells of the transgenic animals listed above. Preferred are transcription regulatory sequences for proteins highly expressed in the salivary gland cells. Introns could be introduced to increase levels of expression. Such introns include the synthetic intron SIS, SV40 small t antigen intron and others (Whitelaw *et al.* 1991; Petitclerc *et al.* 1995).

Preferably, the expression system or construct of this invention also includes a 3' untranslated region downstream of the DNA sequence encoding the desired recombinant protein, or the salivary protein gene used for regulation. This region apparently stabilizes the RNA transcript of the expression system and thus increases the yield of the desired protein. Among the 3' untranslated regions useful in this regard are sequences that provide a polyA signal. Such sequences may be derived, e.g., from the SV 40 small t antigen late polyadenylation signal, synthetic polyadenylation signal or other 3' untranslated sequences well known in this art (Carswell and Alwine 1989;Levitt *et al.* 1989). Preferably, the 3' untranslated region is derived from a salivary-specific protein. The stabilizing effect of this

region's polyA transcript is important in stabilizing the mRNA of the expression sequence. Further, the addition of locus control regions (LCRs), matrix attachment regions (MAR) and scaffold attachment regions (SARs) would allow position-independent, copy number dependent expression of the transgene with either homologous or heterologous promoters (Taboit-Dameron *et al.* 1999;Geyer 1997). Co-integration of an actively expressed gene with the transgene was also shown to increase expression levels of a poorly expressed transgene (Clark *et al.* 1993). Also important in increasing the efficiency of expression of phytase is a strong translation initiation site (Kozak 1987). Likewise, sequences that regulate the post-translational modification of phytase may be useful in the invention.

The term "animal" as used herein denotes all animals except humans. It also includes an individual animal in all stages of development, including embryonic and fetal stages.

A "transgenic" animal is any animal containing cells that bear genetic information received, directly or indirectly, by deliberate genetic manipulation at the subcellular level, such as by microinjection or infection with a recombinant virus. "Transgenic" in the present context does not encompass classical crossbreeding or in vitro fertilization, but rather denotes animals in which one or more cells receive a recombinant DNA molecule. Although it is highly preferred that this molecule be integrated within the animal's chromosomes, the invention also encompasses the use of extrachromosomally replicating DNA sequences, such as might be engineered into yeast artificial chromosomes. The information to be introduced into the animal may be foreign to the species of the animal to which the recipient belongs (i.e., "heterologous"), or the information may be foreign only to the particular individual recipient, or genetic information already possessed by the recipient. In the last case, the introduced gene may be expressed in a manner different than the native gene.

As indicated above, the transgenic animals of this invention are other than human. Farm animals (pigs, goats, sheep, cows, horses, rabbits and the like), rodents (such as mice and rats), domestic pets (eg. cats and dogs), fish and poultry (eg. chickens) are included in the scope of this invention. It is highly preferred that a transgenic animal of the present invention be produced by introducing into single cell embryos appropriate polynucleotides that encode phytase, or fragments or modified products thereof, in a manner such that these polynucleotides are stably integrated into the DNA of germ line cells of the mature animal, and are inherited in normal mendelian fashion. Advances in technologies for embryo micromanipulation now permit introduction of heterologous DNA into fertilized mammalian ova. For instance, totipotent or pluripotent stem cells can be transformed by microinjection, calcium phosphate mediated precipitation, liposome fusion, retroviral infection or other

means, the transformed cells are then introduced into the embryo, and the embryo then develops into a transgenic animal. In one preferred method, developing embryos are infected with a retrovirus containing the desired DNA, and transgenic animals produced from the infected embryo. In a most preferred method, however, the appropriate DNAs are co-injected into the pronucleus or cytoplasm of embryos, preferably at the single cell stage, and the embryos allowed to develop into mature transgenic animals. Such techniques are well known (see reviews of standard laboratory procedures for microinjection of heterologous DNAs into mammalian fertilized ova, including Hogan et al., Manipulating The Mouse Embryo, (Cold Spring Harbor Press 1986); Krimpenfort et al., Bio/Technology 9:844 (1991); Palmiter et al., Cell, 41: 343 (1985); Kraemer et al., Genetic Manipulation Of The Early Mammalian Embryo, (Cold Spring Harbor Laboratory Press 1985); Hammer et al., Nature, 315: 680 (1985); Wagner et al., U.S. Pat. No. 5,175,385; Krimpenfort et al., U.S. Pat. No. 5,175,384, the respective contents of which are incorporated herein by reference).

For a person skilled in art, it will also be clear that the present invention provides for other proteins to be expressed in the salivary gland of the pig. Such proteins may be secreted into saliva to improve digestion and decrease pollution potential (for example, endoglucanases), or specifically targeted for secretion into blood and have effects on the growth and health of the animal (such as growth hormone).

Phytase activity may be measured via a number of assays, the choice of which is not critical to the present invention. For example, the phytase enzyme activity of the transgenic animal tissue may be tested with an ELISA-assay, Western blotting or direct enzyme assays using calorimetric techniques or gel assay system.

The examples included herein are provided so as to give those of ordinary skill in the art a complete disclosure and description of how to make and use the invention and are not intended to limit the scope of what the inventors regard as their invention. Efforts have been made to ensure accuracy with respect to numbers used (e.g., amounts, temperature, pH, etc.) but some experimental errors and deviation should be accounted for. Unless indicated otherwise, temperature is in degrees Centigrade and pressure is at or near atmospheric.

Although the invention has been described with reference to certain specific embodiments, various modifications thereof will be apparent to those skilled in the art without departing from the spirit and scope of the invention as outlined in the claims appended hereto.

Table 1. Secretion of phytase in the saliva of transgenic mice containing the R15-PRP/APPA transgene and non-transgenic mice induced with isoproterenol and pilocarpine.

Founder	Mice	PCR	Gender	Generation	Transgene	Phytase activity micromoles/min/ml
A0m	4bfr (+)	positive	F	1	APPA+intron	39.73
A0m	2brm(+)	positive	M	1	APPA+intron	24.29
A0m	2brm(+)	positive	M	2	APPA+intron	14.42
A0m	5brf(+)	positive	F	2	APPA+intron	7.36
A0m	1brm(-)	negative	M	1	APPA+intron	0.00
A1f	9brf(+)	positive	F	1	APPA+intron	0.08
A1f	11w f(+)	positive	F	1	APPA+intron	0.07
A1f	5brm(+)	positive	M	1	APPA+intron	0.03
A1f	10wf(-)	negative	F	1	APPA+intron	0.02
A20f	1brm(+)	positive	M	1	APPA+intron	0.53
A20f	5brf(+)	positive	F	1	APPA+intron	0.12
A20f	4brf (-)	negative	F	1	APPA+intron	0.03
A2m	13wf(+)	positive	F	1	APPA+intron	87.70
B0m	5brf (+)	positive	F	1	APPA+intron	0.95
B0m	3brm(+)	positive	M	1	APPA+intron	0.73
B0m	6wf (-)	negative	F	1	APPA+intron	0.00
B0f	3wf (+)	positive	F	2	APPA	252.43
B0m-intr	9wf(+)	positive	F	1	APPA	546.74
W0m	8wf(+)	positive	F	1	APPA	60.42
W30m	1wm(+)	positive	M	2	APPA	41.91
W30m	11w f(+)	positive	F	1	APPA	43.44
W30m	4wm(-)	negative	M	1	APPA	0.02
W30m	10wf (-)	negative	F	1	APPA	0.02

Table 2. Repeat sequences found in the Lama2-APPA construct.

Start	End	DNA strand	Repeat	Class/family	Substitutions % of consensus	Deletions % of consensus	Insertions % of consensus
765	927	+	L1M1	LINE/L1	25	4.2	6.7
928	965	+	(CA)n	Simple repeat	0	0	0
966	1020	+	L1M1	LINE/L1	25	4.2	6.7
1021	1156	+	B1_MM	SINE/Alu	15.4	0	0
1159	1231	+	CAAAC)n	Simple repeat	1.4	0	0
1232	1385	+	L1M1	LINE/L1	25	4.2	6.7
1652	2308	C	L1	LINE/L1	28.5	11.9	1.7
2334	2406	C	MIR	SINE/MIR	27.4	4.1	0
2415	3266	+	RMER13A	LTR	17.7	4	6.1
6016	6127	C	L1MA9	LINE/L1	25.5	2	1
6831	7007	+	CT-rich	Low complexity	30.5	1.7	3.4
7299	7510	C	B3	SINE/B2	27.8	7.5	1.4
7718	7746	+	(TCTCTG)n	Simple repeat	6.9	0	0
8499	8581	C	MIR	SINE/MIR	24.1	12.1	3.6
9010	9603	+	Lx4	LINE/L1	21.7	6.4	0.2
10465	10519	+	(TG)n	Simple repeat	5.5	1.8	0
11235	11287	C	MER5A	DNA/MER1 type	28.3	0	1.9
12372	12537	C	L1MA4A	LINE/L1	28.3	5.4	0
14240	14388	+	B1_MM	SINE/Alu	4	0	1.3
14869	14945	C	MIR	SINE/MIR	36.4	1.3	0
16391	16540	C	ORR1D	LTR/MaLR	29.3	0	6
16774	17214	+	RMER4	LTR	21.3	10	11.8
17229	17718	C	L1_MM	LINE/L1	15.3	0	0.8

Table 3. Salivary phytase activities of G2 mice from the founder female 3-1 generated using the construct Lama2-APPA. The mice were between 21 and 30 days of age.

male mouse #	Phytase (U/ml)	female mouse #	Phytase (U/ml)
5	28.3	1	9.0
6	2.5	2	29.9
8	6.6	4	8.0
9	44.7	5	43.0
10	12.7	6	26.9
12	28.3	8	1.9
15	28.1	9	66.3
18	71.2	10	19.9
19	19.5	11	61.3
20	15.7	12	36.4
21	20.9	13	18.0
22	4.1	17	38.9
24	13.0	18	18.5
26	53.4	19	27.0
28	20.4	23	6.5
29	34.1	24	16.1
30	11.1	25	9.4
32	3.1	26	14.8
33	51.7	27	1.3
34	19.0	28	8.2

Table 4. Composition and nutrient levels of Phase II starter diet and low phytate starter diets fed to weanling pigs between 5-10 kg.

Ingredients	Diet/Nutrient Levels[1]	
	Phase II Starter Diet	Low Phytate Starter Diet
Corn	33.15	25.44
Barley	8.00	8.00
Wheat	20.00	40.00
Soybean meal	21.00	8.00
Fish meal	5.00	5.00
Meat and bone meal	-	1.00
Whey	8.00	8.00
Fat	2.00	2.00
Lysine-HCl	0.10	0.28
Dicalcium phosphate	1.10	-
$CaCO_3$	0.90	1.10
Iodized salt	0.30	0.30
Vitamin premix[1]	0.250	0.55
Mineral premix[1]	0.10	0.10
Lincommix 44	0.10	0.10
Total (kg)	100.00	100.00
Calculated nutritive values		
DE (kcal/g)	3.44	3.36
CP (%)	19.46	18.62
Ca (%)	1.00	0.94
Total P (%)	0.74	0.66
Ca/P	1.35:1	1.42:1
Total AA contents (%)		
Arginine	1.16	1.17
Histidine	0.50	0.48
Isoleucine	0.81	0.77
Leucine	1.58	1.54
Lysine	1.17	1.06
Methionine	0.34	0.29
Cysteine	0.34	0.34
Methionine+Cysteine	0.68	0.63
Phenylalanine	0.90	0.90
Tyrosine	0.65	0.65
Threonine	0.75	0.68
Tryptophan	0.23	0.23
Valine	0.91	0.86

[1]Minerals and vitamins meet or exceed levels recommended by NRC (1998).

Table 5. Composition and nutrient levels of grower and finisher diets.

Ingredients	Diet/Nutrient Levels	
	Grower Diet For pigs 20 to 50 kg	Finishing Diet For pigs 50 to 120 kg
Corn	51.78	40.00
Barley	8.10	23.03
Wheat	20.00	23.00
Soybean meal	16.00	13.00
Fat	1.00	1.00
Lysine-HCl	0.12	0.12
Dicalcium phosphate	1.20	1.00
$CaCO_3$	1.15	1.15
Iodized salt	0.50	0.50
Vitamin premix[1]	0.15	0.15
Mineral premix[1]	0.10	0.10
Total (kg)	100.00	100.05
Calculated nutritive values		
DE (kcal/g)	3.39	3.33
CP (%)	14.76	14.17
Ca (%)	0.79	0.74
Total P (%)	0.57	0.53
Ca/P	1.39:1	1.39:1
Total AA contents (%)		
Arginine	0.86	0.80
Histidine	0.38	0.36
Isoleucine	0.58	0.55
Leucine	1.28	1.18
Lysine	0.78	0.73
Methionine	0.24	0.23
Cysteine	0.29	0.29
Methionine+Cysteine	0.53	0.52
Phenylalanine	0.70	0.68
Tyrosine	0.50	0.46
Threonine	0.52	0.49
Tryptophan	0.17	0.16
Valine	0.68	0.65

[1]Minerals and vitamins meet or exceed levels recommended by NRC (1998).

Table 6. Vitamin premix composition[1]

Nutrient	Amount per 5 kg of premix
Wheat midds	3.867 kg
Vitamin A	10 million IU
Vitamin D	1 million IU
Vitamin E	40 thousand IU
Menadione	2.5 g
Pantothenic acid	15 g
Riboflavin	5 g
Folic acid	2 g
Niacin	25 g
Thiamin	1.5 g
Pyridoxine	1.5 g
Vitamin B_{12}	25 mg
Biotin	200 mg
Choline	500 g

[1]From Hoffman-LaRoche Limited, P.O. Box 877, Cambridge, ON. N1R5X9

Table 7. Composition of the mineral premix[1,2]

Mineral component	Amount (%)
Limestone	43.3
Copper sulfate (25%)	6.0
Ferrous sulfate (30%)	33.4
Zinc oxide (72%)	13.9
Manganous oxide (56%)	3.4

[1]Mineral premix prepared at Arkell

[2]Dicalcium phosphate contained 18.5% calcium and 20.5% of phosphate and normally is added at a level of 1.2% to the pig grower diet, 1.0% to the finisher diet and 1.5% to the nursing sow diet.

Table 8. Statistics on embryo recovery and the introduction of embryos containing the transgene into recipient sows.

Treatment	Number
Gilts used for embryo recovery:	
Yorkshire	279
Yorkshire x Landrace cross	168
Duroc	12
Total	459
Recipient sows[1]	74
Embryos transferred to recipients:	
Embryos microinjected with the transgene	4147
Uninjected carrier embryos	675
Total	4543
Total number of embryo transfers	140

[1]Sows were used for up to three farrowings of potentially transgenic pigs. Sows were inseminated with Yorkshire semen from a high breeding value boars.

Table 9. Transgenic pigs containing a salivary phytase gene generated by microinjections of single cell zygotes using the Lama2-APPA transgene

ID # of pig[1]	Birth Date	Presence of Transgene[2] Tail/Blood	Sex	Salivary phytase (U/ml)[3]	Zygote source[4]
167-02	Apr 14/99	+/+	Boar	6,000	Yorkshire
282-02	Jun 14/99	+/+	Boar	618	Yorkshire
282-04	Jun 14/99	+/+	Boar	1,349	Yorkshire
405-02	Aug 14/99	+/+	Gilt	339	York/Landrace
421-02	Aug 24/99	-/+	Gilt	0.8	York/Landrace
421-04	Aug24/99	-/+	Gilt	2.2	York/Landrace
421-06	Aug 24/99	+/+	Boar	97	York/Landrace
448-01	Sep 03/99	+/+	Gilt	0	York/Landrace
491-01	Sep 25/99	+/+	Gilt	2.3	York/Landrace
491-02	Sep 25/99	+/+	Gilt	0	York/Landrace
491-03	Sep 25/99	+/+	Gilt	0.3	York/Landrace
491-05	Sep 25/99	+/+	Boar	0	York/Landrace
496-05	Sep 26/99	+/+	Boar	0	York/Landrace
500-03	Sep 28/99	+/+	Boar	136	York/Landrace
510-01	Sep 28/99	+/+	Boar	0.2	York/
559-05	Nov 01/99	+*/+	Boar	>418	York/Landrace
560-04	Nov 02/99	+*/+	Boar	5	Yorkshire
594-03	Nov 18/99	+/+	Gilt	2.3	Yorkshire
613-02	Nov 27/99	-/+	Gilt	0.5	York/Landrace
613-03	Nov 27/99	-/+	Gilt	0.3	York/Landrace
647-01	Dec 13/99	-/+	Gilt	0.5	York/Landrace
647-03	Dec 13/99	+*/+	Gilt	16.3	York/Landrace
647-04	Dec 13/99	-*/+	Gilt	0.5	York/Landrace
647-08	Dec 13/99	-*/+	Boar	0.4	York/Landrace
647-09	Dec 13/99	+*/+	Boar	1.92	York/Landrace
668-01	Dec 17/99	+*/+	Gilt	489	Yorkshire
671-02	Dec 19/99	+*/+	Boar	6.9	York/Landrace
671-04	Dec 19/99	+*/+	Boar	325	York/Landrace
675-03	Dec 21/99	-*/+	Gilt	2.1	York/Landrace
675-04	Dec 21/99	+*/+	Boar	42.6	York/Landrace
675-06	Dec 21/99	-*/+	Boar	5.0	York/Landrace

[1]The number preceeding the dash represents the litter number and the number following the dash is the pig number within the litter.
[2]All PCR assays were conducted with the primer APPA-up2-APPA-Kpn. Assays indicated with a star gave a negative result with the primer pair. However these samples gave a positive result for the primer set APPA-d4-Lama-up1. Samples 613-02 and 613-03 were negative with the latter primer set.
[3]Saliva was sampled and assayed for phytase 2 to 4 days after birth of the piglets.
[4]Zygotes used for microinjection were collected from superovulated Yorkshire or Yorkshire-Landrace cross gilts.

Table 10. Phosphorus content of feces collected from pigs producing a salivary phytase and non-transgenic pen-mates[1]. The data was subjected to a T-test analysis and the data recorded below.

	Mean Fecal Phosphorus (%)	SE	Relative reduction in fecal phosphorus (%)	t	t (1%)
1. 167-02 Grower Diet (122 days):	1.59		24.47		
Non-transgenic (n=4)	2.11	0.0604669		8.517	4.6
2. 167-02 Finisher Diet (154 days):	1.97		16.97		
Non-transgenic (n=4)	2.37	0.0240767		16.717	4.6
3. 282-02 Grower Diet (93 days):	1.85		12.90		
Non-transgenic (n=5)	2.124	0.022231964		12.324	4.03
4. 282-02 Finisher Diet (145 days):	1.76		16.03		
Non-transgenic (n=5)	2.096	0.099153384		3.389	4.03[2]
5. 282-04 Grower Diet (93 days):	1.95		8.19		
Non-transgenic (n=5)	2.124	0.022231964		7.827	4.03
6. 282-04 Finisher Diet (145 days):	1.56		25.57		
Non-transgenic (n=5)	2.096	0.099153384		5.406	4.03
7. 421-06 Starter II Diet (40 days):	1.17		27.47		
Non-transgenic (n=5)	1.612	0.086155741		5.140	4.03
8. 421-06 Start III Diet (48 days):	1.57		18.01		
Non-transgenic (n=5)	1.915	0.102884789		3.351	4.03
9. 421-06 Grower Diet (81 days):	2.00		13.28		
Non-transgenic (n=5)	2.310	0.151658823		2.022	4.03
10. 421-06 Finisher Diet (136 days):	1.71		21.20		
Non-transgenic (n=5)	2.173	0.053023237		8.687	4.03
11. 405-02 Starter II Diet (40 days):	1.81		26.97		
Non-transgenic (n=5)	2.482	0.173625623		3.856	4.03
12. 405-02 Starter III Diet (48 days):	1.54		36.58		
Non transgenic (n=4)	2.430	0.104642248		8.496	4.6
13. 405-02 Grower Diet (80 days):	2.26		18.19		
Non-transgenic (n=4)	2.763	0.124724697		4.029	4.6
14. 405-02 Finisher Diet (136 days):	2.26		13.24		
Non-transgenic (n=4)	2.605	0.217198066		1.588	4.6

[1]Fresh fecal samples were collected on 3 different days was freeze-dried and then dried to constant weight at 110°C for 24 h, and analyzed for total phosphorus.
[2]At the 5% level of confidence t=2.57.

Table 11. Phytase activities of the first generation (G1) transgenic offspring obtained by the crossing the phytase positive boar 167-02 with non-transgenic Yorkshire gilts[1]

ID # of pig	Birth Date	Sex	Salivary phytase (U/ml)	Specific Activity U/mg protein
151-01	Mar 16/00	F	1193	126
151-02	"	F	736	63.3
151-05	"	M	710	109
151-07	"	M	8019	315
152-04	"	M	10077	364
152-09	"	M	3054	200
154-01	Mar 19/00	F	2472	256
154-03	"	F	6425	706
154-04	"	F	n.d.	n.d.
154-05	"	M	2767	213
154-06	"	M	341	39
154-07	"	M	4029	142
154-08	"	M	1184	47.4
159-03	Mar 20/00	F	1563	116
159-04	"	M	2285	201

[1]The number of males and females (M/F) in each litter were 5/3, 7/2, 5/4, and 2/3 for litter numbers 151, 152, 154 and 159, respectively. Saliva was collected from the piglets on day 11.

Table 12. Primers used for construction and detection of transgenic constructs.

Name	Start-End[1]	Forward/ Reverse	
Primers used in R15/APPA+intron and R15/APPA construction			
APPA-DOWN2		R	TCGGCGCTCACCTTGAGTTC
APPA-DRA		F	CCGTTTAAAGCCATCTTAATCCCAT
APPA-SMA		R	GTCCCGGGTATGCGTGCTTCATTC
CAT-ATG		R	CCATGGTGGCGGCTTTTAGCTTCCTTAGCT CCTGA
CAT-TAA		F	AGCGCTTGCAGTTTGTAAGGCAGTTATTG GTGCCC
CAT-UP1		F	TCG AGG AGC TTG GCG AGA TT
R15-UP1		F	TTTCGGGCCAATGTTGCTGT
Primers used in SV40/APPA+intron construction			
SV-HIND		F	CCCAAGCTTTACACTTTATGC
SV-XHO		R	GCCCTCGAGCCTCCTCACTACTTCT
Primers used in Lama2/APPA and Lama2/PSP/APPA construction			
APPA-CLA	12635-12657	F	GGATCGATAAAAGCCGCCACCATGAA
APPA-DOWN2	13307-13326	R	TCGGCGCTCACCTTGAGTTC
APPA-DOWN4	12751-12780	R	GCACGCACACCATGACGACTGACAATCAC C
APPA-KPN	13935-13959	R	CGGGTACCTTACAAACTGCAAGCGG
APPA-MATURE	12719-12738	F	CAGAGTGAGCCGGAGCTGAA
APPA-UP2	13210-13229	F	CGAACTGGAACGGGTGCTTA
LAMA-CLA	12615-12639	R	GCATCGATCTTTGGTTCTGACAAATGG
LAMA-SIGNAL		R	TGACTCTGAGTTCCCAATGA
LAMA-UP	12111-12130	F	GTGCTGCTCCAAGTTTGGTG
Primers for detection of the porcine β-globin gene			
PIG-BGF		F	GCAGATTCCCAAACCTTCGCAGAG
PIG-BGR		R	TCTGCCCAAGTCCTAAATGTGCGT

1 The location of the primers shown for Lama2/APPA sequence.
The start and stop codons of *APPA* are indicated in bold letters, the optimal initiation sequence for translation is italicized, and the restriction sites for restriction enzymes are underlined.

Reference List

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THE EMBODIMENTS OF THE INVENTION IN WHICH AN EXCLUSIVE PROPERTY OR PRIVILEGE IS CLAIMED ARE DEFINED AS FOLLOWS:

1. A transgenic non-human animal that carries in the genome of its somatic and germ cells a transgene construct comprising (a) a trangene encoding a protein operably linked to (b) a first regulatory sequence for salivary gland specific expression of said protein, wherein said animal is selected from the group consisting of pigs, goats, sheep, cows, horses, fish and poultry.

2. The animal of claim 1 wherein said first regulatory sequence comprises a salivary protein promoter/enhancer sequence, whereby said animal expresses said protein in its salivary glands.

3. The animal of claim 2 wherein said saliva protein promoter/enhancer sequence comprises a parotid secretory protein (PSP) promoter/enhancer, a proline-rich protein (PRP) promoter/enhancer or a salivary amylase promoter/enhancer.

4. The animal of claim 3 wherein said promoter/enhancer is a parotid secretory protein (PSP) promoter/enhancer.

5. The animal of claim 4 wherein said parotid secretory protein (PSP) promoter/enhancer is derived from a mouse.

6. The animal of claim 3 wherein said promoter/enhancer is a proline-rich protein (PRP) promoter/enhancer.

7. The animal of claim 6 wherein said proline-rich protein (PRP) promoter/enhancer is derived from a rat.

8. The animal of claim 1 wherein said transgene is further operably linked to (c) one or more second regulatory sequences including enhancers, transcription regulatory sequences, termination sequences, and polyadenylation sites.

9. The animal of any one of claims 1 to 8 wherein said animal is a pig.

10. The animal of any one of claims 1 to 9 wherein said protein is a phytase.

11. The animal of any one of claims 1 to 10 wherein said animal is a pig, said protein is a phytase and said first regulatory sequence comprises a parotid secretory protein (PSP) promoter/enhancer or a proline-rich protein (PRP) promoter/enhancer.

12. The animal of any one of claims 1 to 11 wherein said transgene construct comprises a nucleic acid sequence according to SEQ ID NO:3, SEQ ID NO:5; or SEQ ID NO:7.

13. A transgenic non-human animal that carries in the genome of its somatic and germ cells a transgene construct, said construct comprising a trangene encoding phytase, wherein said animal is selected from the group consisting of pigs, goats, sheep, cows, horses, fish and poultry.

14. An animal according to claim 13 wherein said phytase is *Escherichia coli AppA* phytase.

15. The animal of claim 13 or 14 wherein said transgene is operably linked to a first regulatory sequence for salivary gland specific expression of said phytase.

16. The animal of claim 15 wherein said first regulatory sequence comprises a parotid secretory protein (PSP) promoter/enhancer, a proline-rich protein (PRP) promoter/enhancer or a salivary amylase promoter/enhancer.

17. The animal of claim 13 wherein said phytase is expressed in saliva or in the gastrointestinal tract of said animal.

18. The animal of claim 13 wherein said transgene construct comprises a nucleic acid sequence according to SEQ ID NO:3, SEQ ID NO:5; or SEQ ID NO:7.

19. A method of expressing a protein in the gastrointestinal tract of an animal, the method comprising the steps of:

a) introducing a transgene construct into a non-human animal embryo such that a non-human transgenic animal that develops from said embryo has a genome that comprises said transgene construct, wherein said transgene construct comprises:

i) a transgene encoding said protein, and

ii) at least one regulatory sequence for gastrointestinal tract specific expression of said protein,

b) transferring said embryo to a foster female; and,

c) developing said embryo into said transgenic animal

wherein said transgene is produced in the gastrointestinal tract of said animal, wherein said animal is selected from the group consisting of pigs, goats, sheep, cows, horses, fish and poultry.

20. The method of claim 19 wherein said regulatory sequence provides for salivary gland or pancreatic gland specific expression of said protein.

21. The method of claim 19 wherein said regulatory sequence provides for salivary gland specific expression of said protein.

22. The method of claim 21 wherein said salivary gland is a parotid gland, submaxillary gland, or a submandibular gland.

23. The method of claim 21 wherein said transgene is expressed in the salivary gland of said animal.

24. The method of claim 19 wherein said at least one regulatory sequence comprises a salivary protein promoter/enhancer sequence.

25. The method of claim 19 wherein said protein is a glycoprotein.

26. The method of claim 19 wherein said protein is a phytase.

27. A method according to claim 26 wherein said phytase is *Escherichia coli AppA* phytase.

28. The method of claim 19 wherein said transgene construct comprises a nucleic acid sequence according to SEQ ID NO:3, SEQ ID NO:5, or SEQ ID NO:7.

29. A transgenic animal prepared according to the method of claim 19, or a progeny thereof.

30. A process for producing a protein comprising the steps of:

a) obtaining salivary gland secretion containing said protein from a non-human transgenic animal, said animal containing within its genome a transgene construct, wherein said transgene construct comprises:

i) a transgene encoding said protein, and

ii) at least one regulatory sequence for salivary gland specific expression of said protein, and

extracting said protein from said saliva.

31. The process of claim 30 wherein said at least one regulatory sequence comprises a salivary protein promoter/enhancer sequence.

32. The process of claim 30 wherein said protein is a glycoprotein.

33. The process of claim 30 wherein said transgene construct comprises a nucleic acid sequence according to SEQ ID NO:3, SEQ ID NO:5; or SEQ ID NO:7.

34. The process of claim 30 wherein said protein is a phytase.

35. The process of claim 30 wherein said salivary gland is a parotid gland, submaxillary, or a submandibular gland.

36. A method for expressing a phytase in a non-human animal, said method comprising:

a) constructing a nucleic acid sequence including a transgene construct comprising:

i) a transgene encoding said phytase, and

ii) at least one regulatory sequence for gastrointestinal tract specific expression of said protein, and

b) transfecting the animal with said nucleic acid sequence;

whereby said animal carries within the genome of its somatic and germ cells said transgene construct and wherein said animal expresses said phytase in its gastrointestinal tract and wherein the animal is selected from the group consisting of pigs, goats, sheep, cows, horses, fish and poultry.

37. The method of claim 36 wherein said transgene construct results in salivary gland or pancreatic gland specific expression of said phytase.

38. The method of claim 37 wherein said regulatory sequence provides for salivary gland specific expression of said phytase.

39. The method of claim 38 wherein said salivary gland is a parotid gland, submaxillary, or a submandibular gland.

40. The method of claim 38 wherein said phytase is expressed in the saliva of said mammal.

41. The method of claim 38 wherein said transgene construct comprises a nucleic acid sequence according to SEQ ID NO:3, SEQ ID NO:5; or SEQ ID NO:7.

42. The method of claim 38 wherein said transgene construct comprises a nucleic acid sequence according to SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:4, or SEQ ID NO:6.

43. The method of claim 38 wherein said animal is a pig.

44. A nucleic acid molecule comprising (a) a nucleic acid sequence encoding a phytase operably linked to (b) at least one regulatory sequence for gastrointestinal tract specific expression of said phytase.

45. The molecule of claim 44 wherein said at least one regulatory sequence comprises a salivary protein promoter/enhancer sequence, whereby expression of said protein is salivary gland specific.

46. The molecule of claim 45 wherein said salivary protein promoter/enhancer sequence comprises a parotid secretory protein (PSP) promoter/enhancer, a proline-rich protein (PRP) promoter/enhancer, a salivary amylase promoter/enhancer, or a SV40 promoter/enhancer.

47. The molecule of claim 44 wherein said molecule comprises a nucleic acid sequence according to SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:4, or SEQ ID NO:6.

48. The molecule of claim 44 wherein said molecule includes a nucleic acid sequence according to SEQ ID NO:3, SEQ ID NO:5; or SEQ ID NO:7.

49. An antibody specific to a protein expressed by a nucleic acid sequence according to SEQ ID NO:3, SEQ ID NO:5; or SEQ ID NO:7.

50. The antibody of claim 49 wherein said antibody is monoclonal.

51. The antibody of claim 49 wherein said antibody is polyclonal.

52. A hybridoma secreting the antibody of claim 50.

53. A host cell transfected with molecule according to any one of claims 44 to 48.

54. The host cell of claim 53 wherein said cell is an animal cell.

55. A diagnostic kit for immunologically detecting a protein expressed by a nucleic acid sequence according to SEQ ID NO:3, SEQ ID NO:5; or SEQ ID NO:7, the kit including an antibody specific to said protein.

56. The kit of claim 55 wherein said antibody is monoclonal.

57. The kit of claim 56 wherein said antibody is polyclonal.

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(54) Title: TRANSGENIC ANIMALS EXPRESSING SALIVARY PROTEINS

(57) Abstract

The invention provides a transgenic animal having within its genome a transgene construct for gastrointestinal tract specific expression of a protein. In a preferred embodiment, the protein is a phytase or a homologue thereof. Such proteins may be heterologous and may be specifically expressed in the salivary gland of the animal by operably linking the nucleic acid sequence encoding the protein with regulatory sequence including a salivary gland protein promoter/enhancer. Also provided are methods of expressing and producing proteins using such nucleic acid constructs. Further, antibodies specific to such proteins and immunological diagnostic kits are also provided.

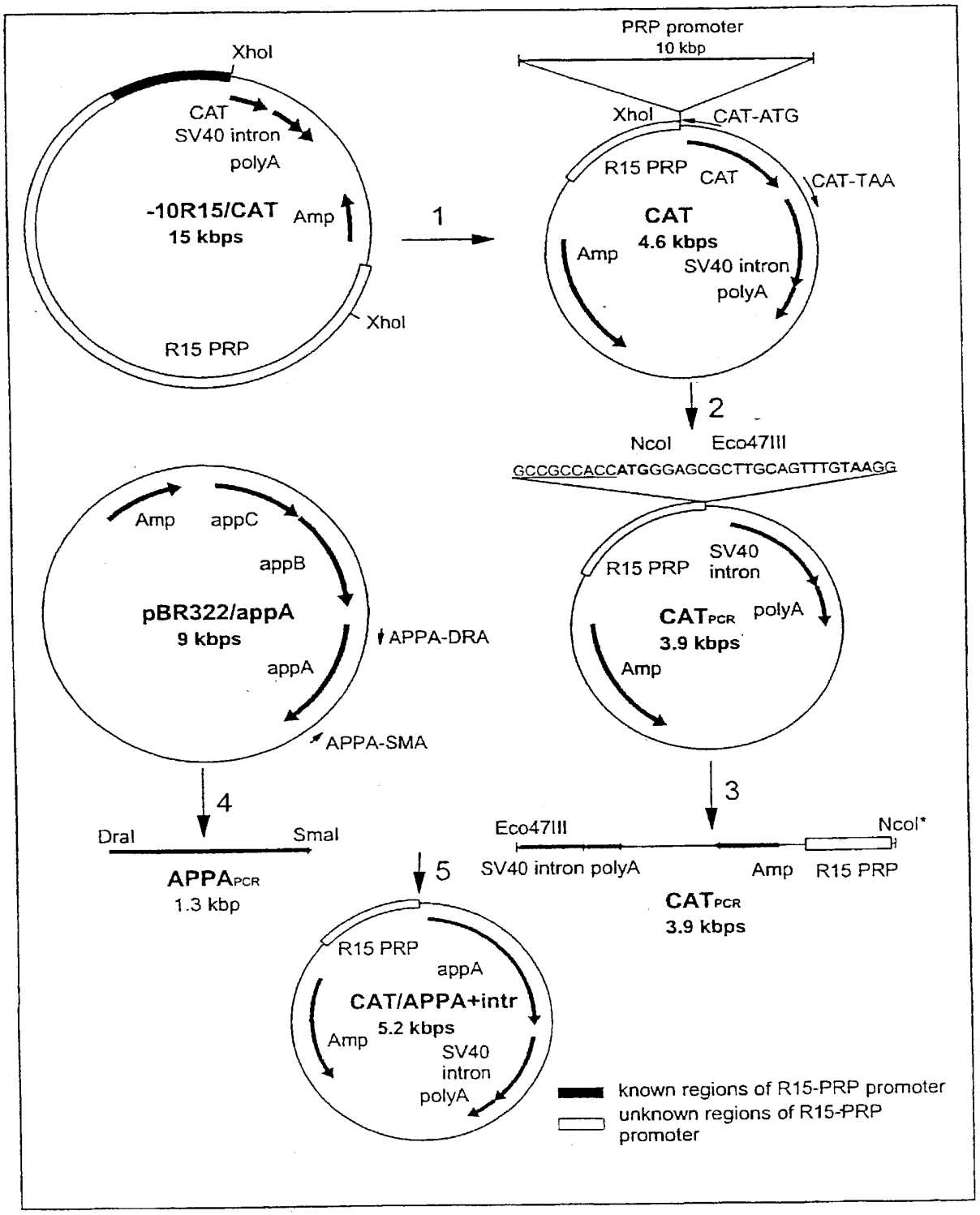
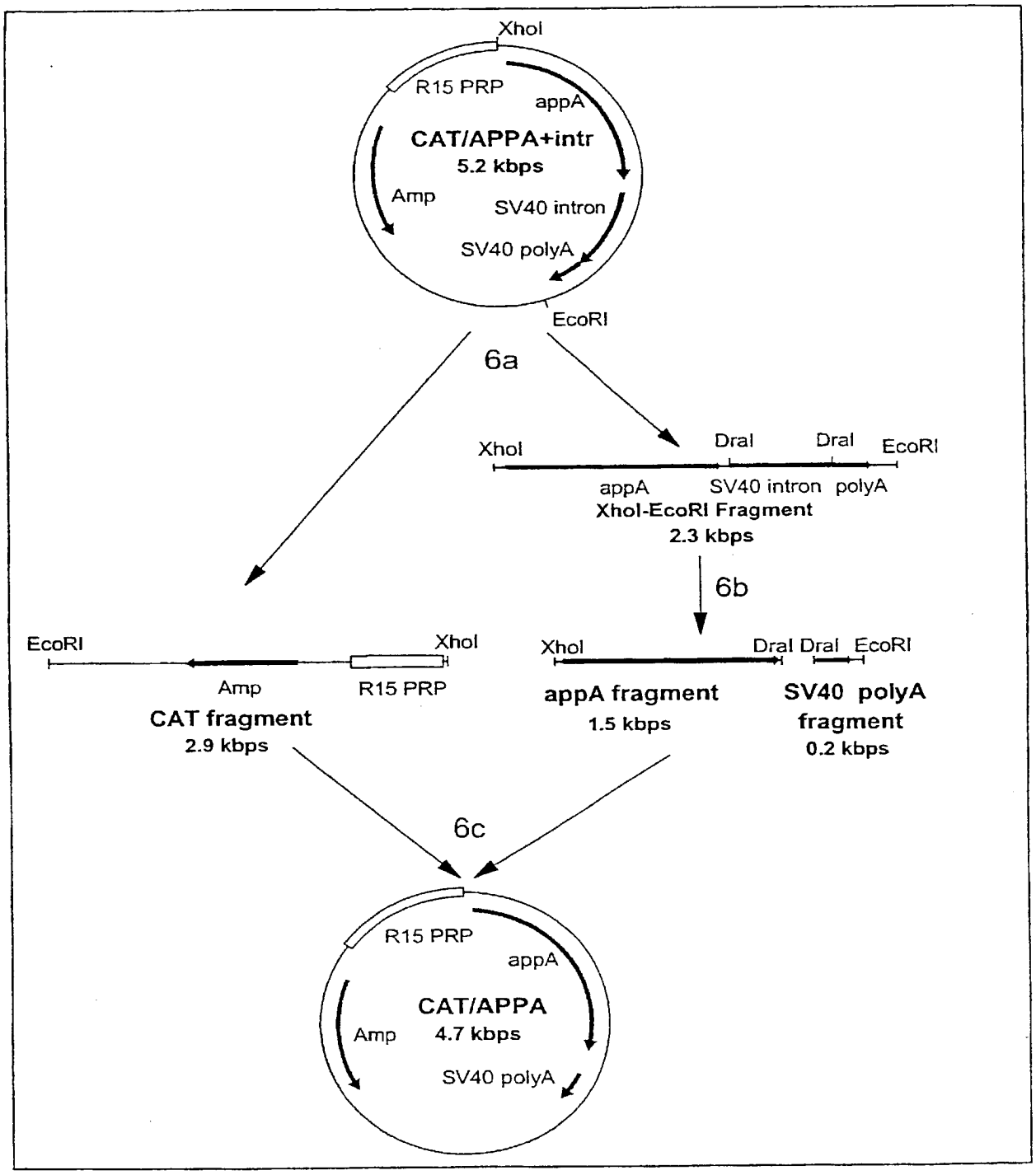


Figure 1



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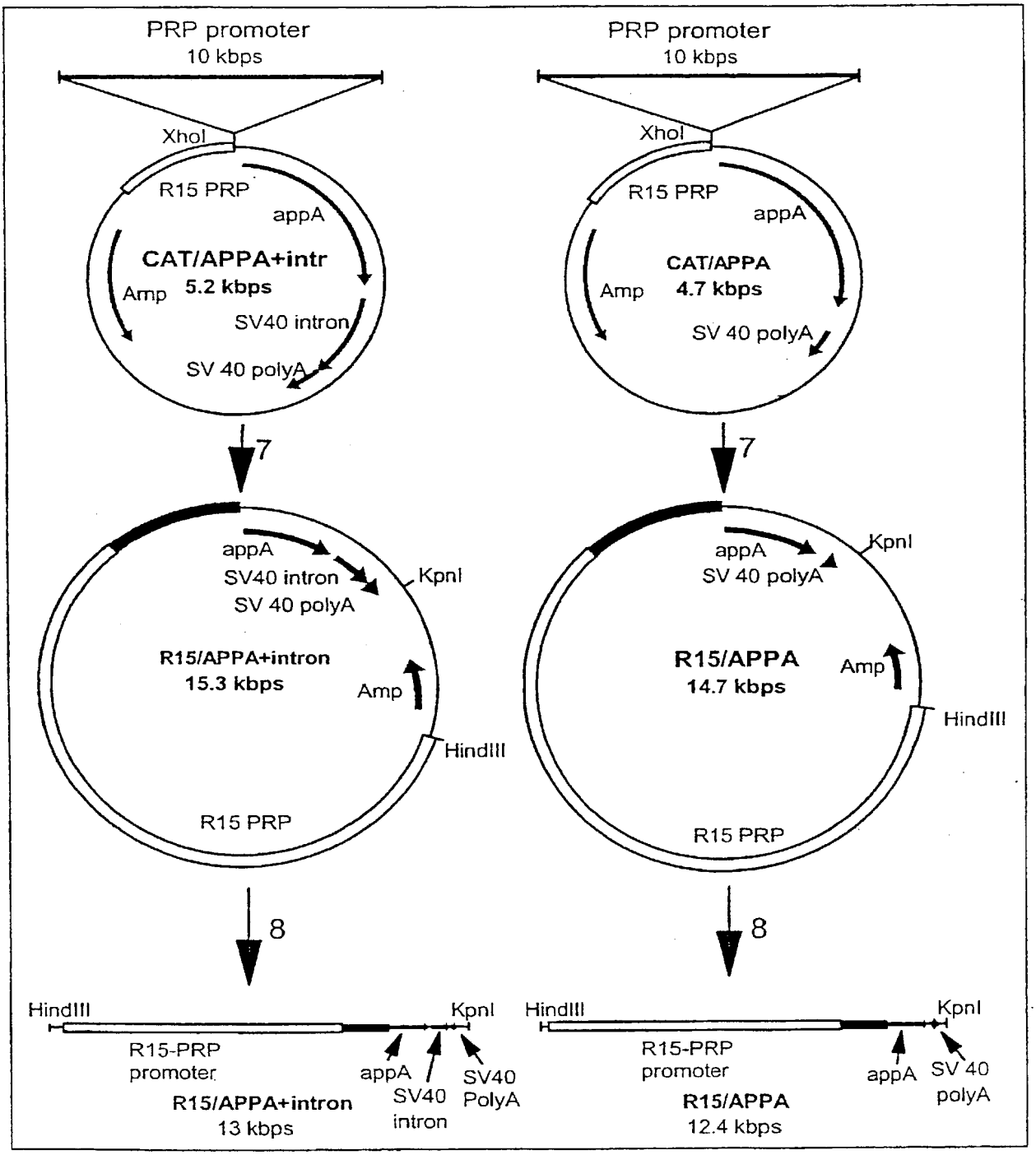

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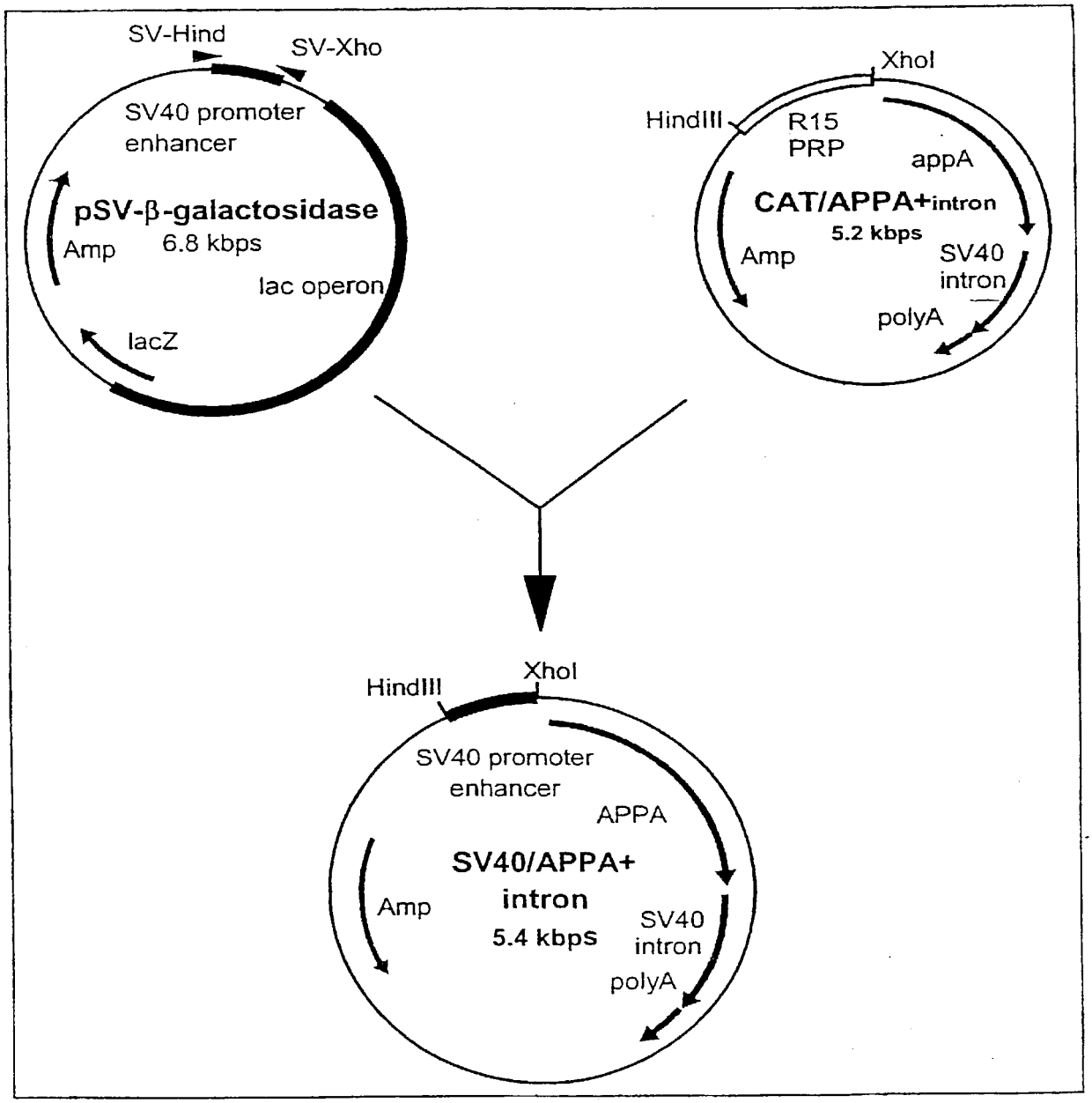
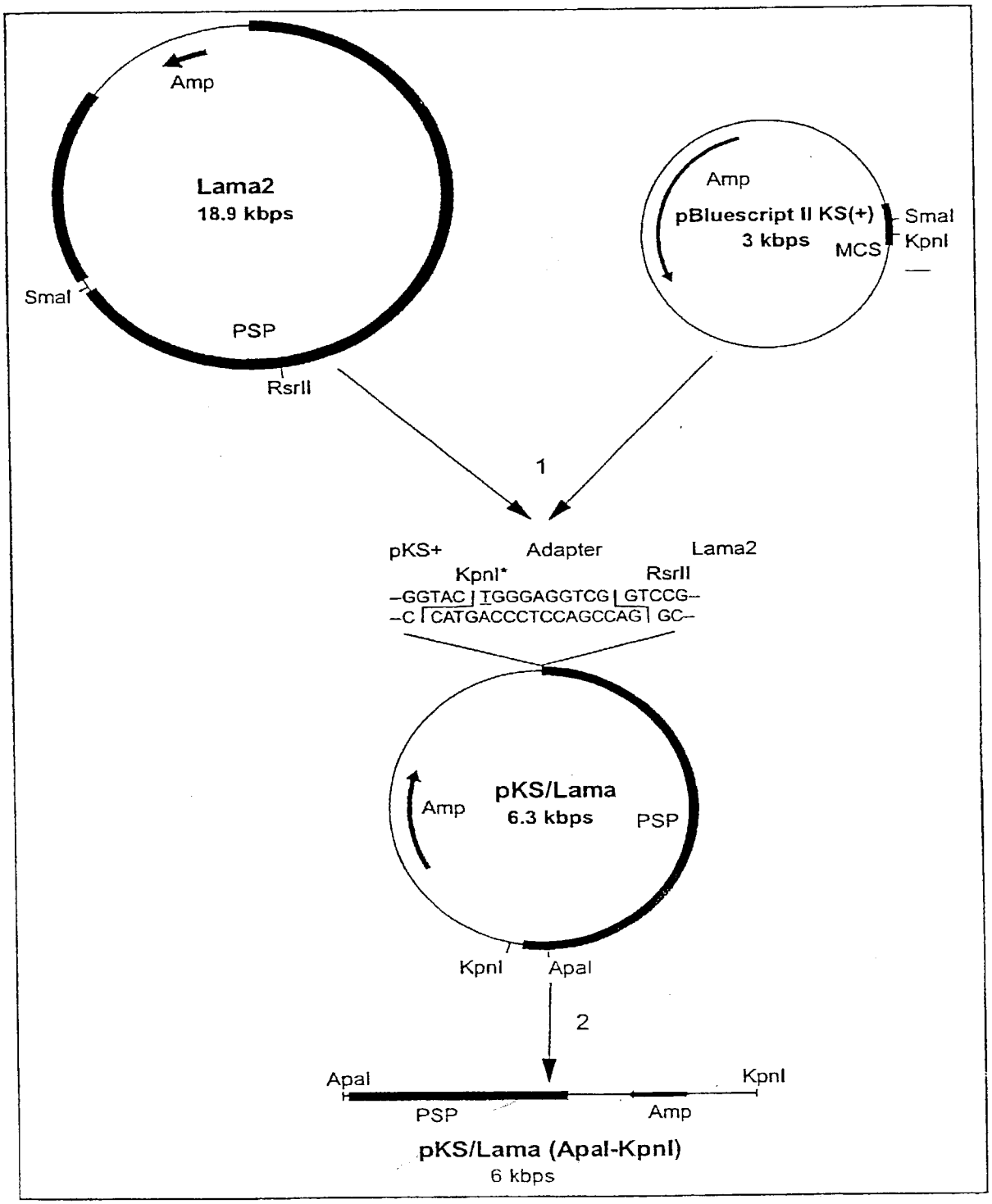


Figure 2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pKS+
Adapter
Lama2
KpnI*
RsrII
–GGTAC⌋IGGGAGGTCG⌊GTCCG–
–C⌈CATGACCCTCCAGCCAG⌉GC–
Amp
pKS/Lama
6.3 kbps
PSP
KpnI
ApaI
2
ApaI
KpnI
PSP
Amp
pKS/Lama (ApaI-KpnI)
6 kbps

Figure 3

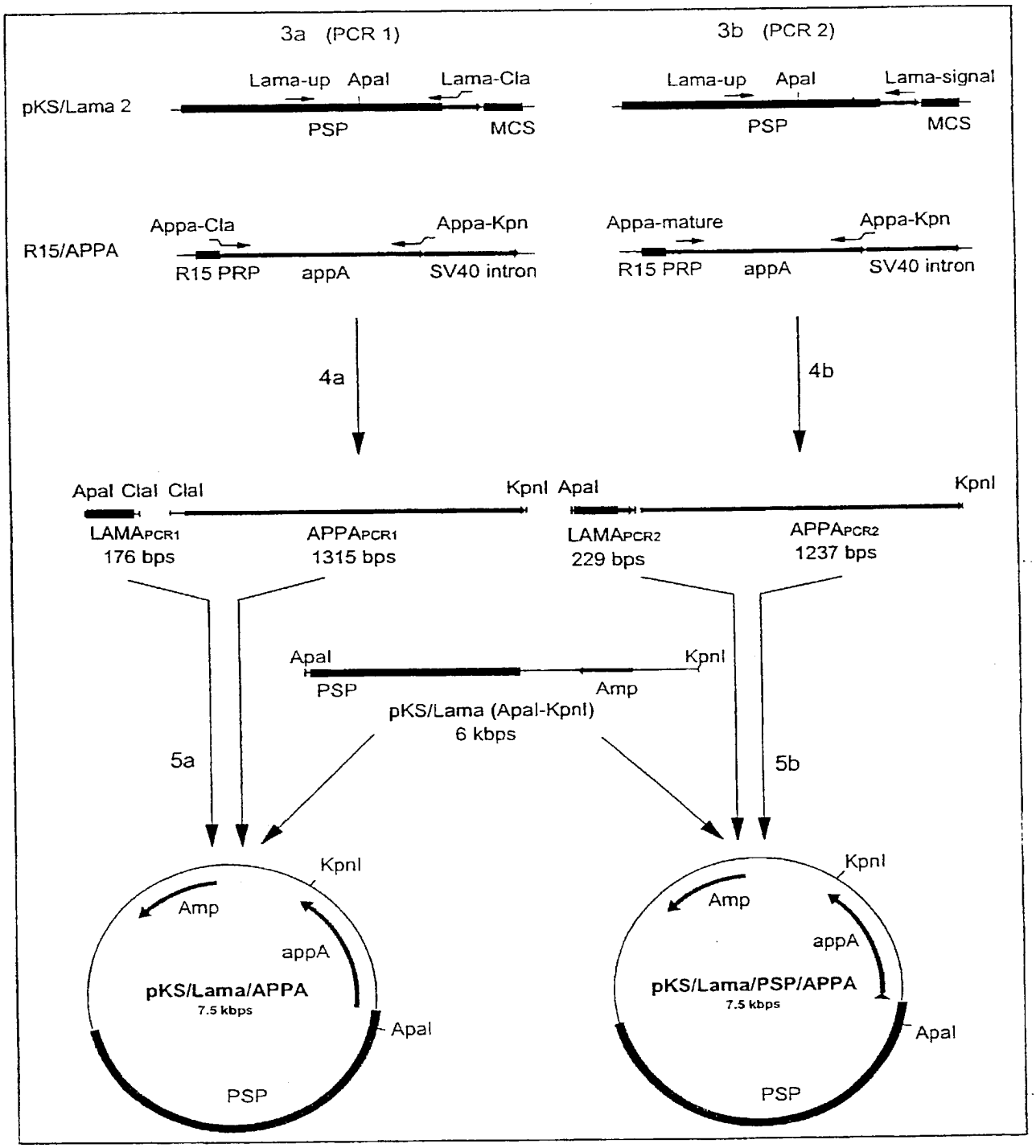

Figure 3 (continued)

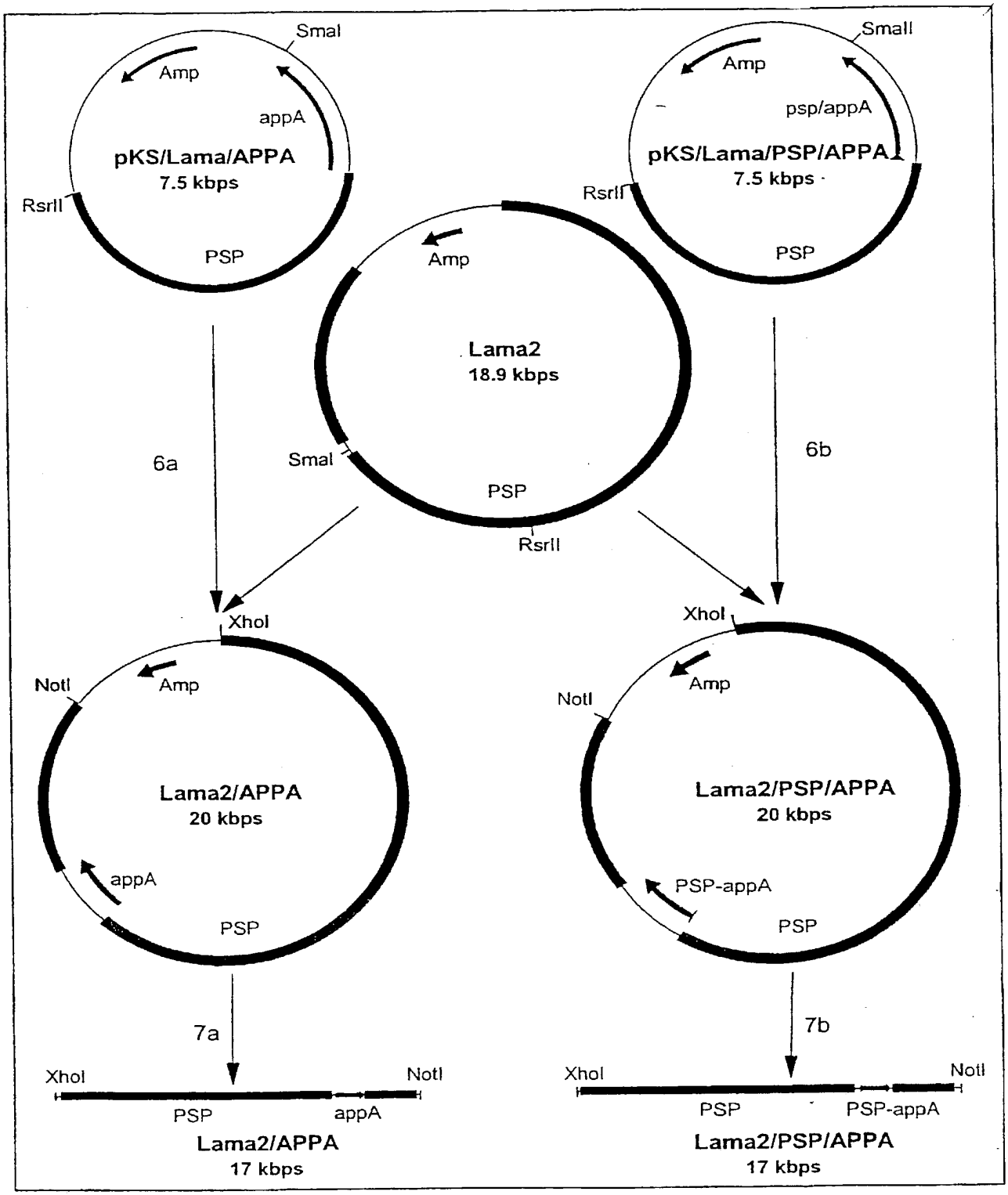

Figure 3 (continued)

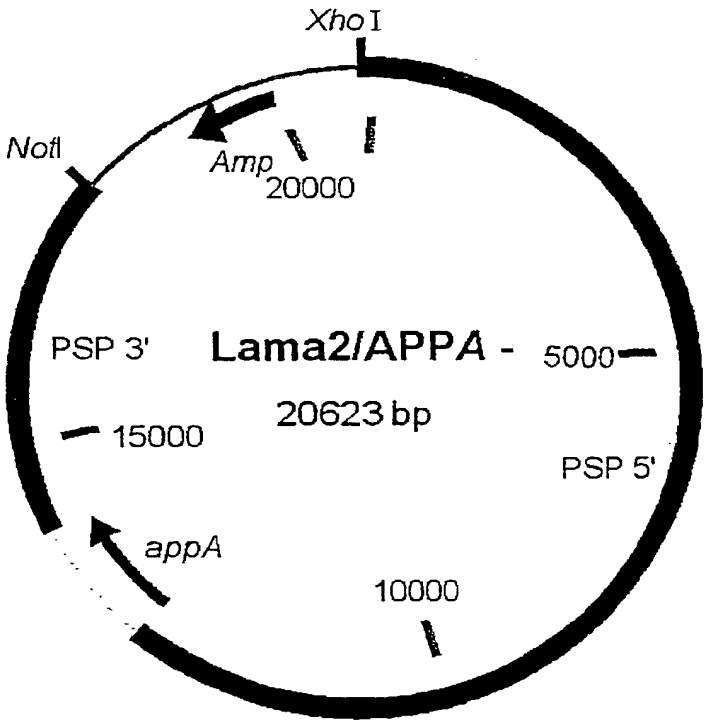

Figure 4. Schematic diagram of the Lama2/APPA construct.

Figure 5. The nucleic acid sequence of the Lama2/APPA plasmid (SEQ ID NO: 1)

```
LOCUS       Lama-appA   20623 bp    DNA   CIRCULAR SYN        17-JAN-2000
DEFINITION  Lama 2/APPA transgenic construct
ACCESSION   Lama 2-appA,
KEYWORDS    parotid secretory protein; acid glucose-1-phosphatase; appA
            gene;
            periplasmic phosphoanhydride phosphohydrolase; artificial
            sequence;
            cloning vector
REFERENCE   1  (bases 1 to 20623)
AUTHORS     Golovan, S., Forsberg, C.W., Phillips, J.
JOURNAL     Unpublished.
FEATURES
DEFINITION  M. musculus Psp gene for parotid secretory protein.
   ACCESSION   X68699
   VERSION     X68699.1  GI:53809
   SOURCE      house mouse.
   ORGANISM  Mus musculus
      Eukaryota; Metazoa; Chordata; Craniata; Vertebrata; Mammalia;
      Eutheria; Rodentia; Sciurognathi; Muridae; Murinae; Mus.
      REFERENCE   1  (bases 3777 to 5332;)
      AUTHORS   Svendsen,P., Laursen,J., Krogh-Pedersen,H. and Hjorth,J.P.
      TITLE     Novel salivary gland specific binding elements located in the PSP
            proximal enhancer core
      JOURNAL   Nucleic Acids Res. 26 (11), 2761-2770 (1998)
      MEDLINE   98256451
      REFERENCE   2  (bases 7147 to 12653; 13952 to 17731)
      AUTHORS   Mikkelsen,T.R.
      TITLE     Direct Submission
      JOURNAL   Submitted (07-OCT-1992) T.R. Mikkelsen, Department of Molecular
                  Biology, University of Aarhus, CF Mollers Alle 130, 8000
                  Aarhus, DENMARK
      REFERENCE  3  (bases 7147 to 12653; 13952 to 17731)
      AUTHORS   Laursen J, Hjorth JP
      TITLE A cassette for high-level expression in the mouse salivary glands.
      JOURNAL   Gene 1997 Oct 1;198(1-2):367-72
      MEDLINE   9370303

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                    regulatory region"
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                    /number=1
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Figure 5 (continued):

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      ACCESSION     M58708 L03370 L03371 L03372 L03373 L03374 L03375
      VERSION       M58708.1  GI:145283
      SOURCE        Escherichia coli DNA.
      ORGANISM      Escherichia coli
            Bacteria; Proteobacteria; gamma subdivision; Enterobacteriaceae;
            Escherichia.

REFERENCE   1  (bases 12653..13951)
      AUTHORS    Dassa,J., Marck,C. and Boquet,P.L.
      TITLE      The complete nucleotide sequence of the Escherichia coli gene appA
                 reveals significant homology between pH 2.5 acid phosphatase
                 and glucose-1-phosphatase
      JOURNAL    J. Bacteriol. 172 (9), 5497-5500 (1990)
      MEDLINE    90368616

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Figure 5 (continued):

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      ACCESSION     X52327
      VERSION       X52327.1  GI:58061
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      SOURCE        synthetic construct.
      ORGANISM      synthetic construct
                    artificial sequence.
      REFERENCE     1       (bases 17732 to 20623)
      AUTHORS       Thomas,E.A.
      TITLE         Direct Submission
      JOURNAL       Submitted (20-FEB-1990) Thomas E.A., Stratagene Cloning
                    Systems, 11099 North Torney Pines Rd., La Jolla, CA 92037, USA
      REFERENCE     2       (bases 17732 to 20623)
      AUTHORS       Short,J.M., Fernandez,J.M., Sorge,J.A. and Huse,W.D.
      TITLE         Lambda ZAP: a bacteriophage lambda expression vector with in
                    vivo excision properties
      JOURNAL       Nucleic Acids Res. 16 (15), 7583-7600 (1988)
      MEDLINE       88319944
      REFERENCE     3       (bases 17732 to 20623)
      AUTHORS       Alting-Mees,M.A. and Short,J.M.
      TITLE         pBluescript II: gene mapping vectors
      JOURNAL       Nucleic Acids Res. 17 (22), 9494 (1989)
      MEDLINE       90067967
      FEATURES              Location/Qualifiers
      Source                17732 to 20623
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      CDS                   complement (18967..19827)
                    /gene="Amp"
                    /product="b-lactamase"

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      121 TGTTGAACAA GTTCTCCAAA GGAGAGATAC AGATGAGTGC GTATAGGGTG GACCTGGCTG
      181 CTGAGGAGAC ACCTGCATCT GACTAAGAAG AGCCACGGTG TTAGTTGAAT GGTGTGGAGT
      241 AGGGTGGTTC TGTGGGACAG TAGAAAATCG AGAGGCATGT GCCGTTTAGT GAACTGATGG
      301 AAGCTACCCC AAACGACAGA GATTGTCAGT CAGGCCAATC CGTTTCGAGT TTGATGGGCA
      361 GCCGGACAGT GAGACAGACA CACCTACTCA GTTGGAGGAA GGATGAGAAC AATGGCCAGC
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      481 ACAAAGCTGC CAAAGACCAA AGACTTGTTC TCCATTAGAA ATGACAGCTG GCTTGACCCG
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      841 CACCAAGACT GCAGCACACC CCTGTCAGAT GGCTGTGATC AAGAAAATAA ATGACAATGA
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     1321 ACTACACTGT TCACCACAGC CAGGCTGTGG AACCAGCCTG AGTGTCCATG ATAAATGAAT
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Figure 5 (continued):

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Figure 5 (continued):

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9361 GGTCCGAGGC ACCTGGCACG TGTGAAGCAG ACATGCAGCT CAGTCTCCAT GTAGGTCCTC
9421 CAATAAGCGG TAGCCTGACT GCAGTATCCA ATCCCCAACA GGGCTGCATA GTCTGGCCTC
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Figure 5 (continued):

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 9721 TTTCATGGGC CTAGGACTTG GTGACCAGTT CAAACAATGG GGCCTGTGGA AGACACAATA
 9781 TACAAGACTA GGGACATTCC TGTTCTGCTG ACTATCCATA GCCTGATGTA GGTGGAAGGA
 9841 CCCAATCACT GGATTTCTAC CCTTGCACAA CCTTGACAGC TGAGGGCCTC TCAGAAACCT
 9901 ATTTCTTCCA CTGAAAAATG AGACTCTCAA ATGAACGTCG TGACAATCAT CAGGCTTATT
 9961 AAAGAGGTGT ATCTAACCTG AATGGCAAGC AGACAGCAGG CAAATGTCTG TATCAACCTC
10021 TAGGAAGGAC AAGAACTGCT CACTGCTGCC CCCCAGGAGG CCATTTGCTG AAACAGCTGC
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Figure 5 (continued):

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Figure 5 (continued):

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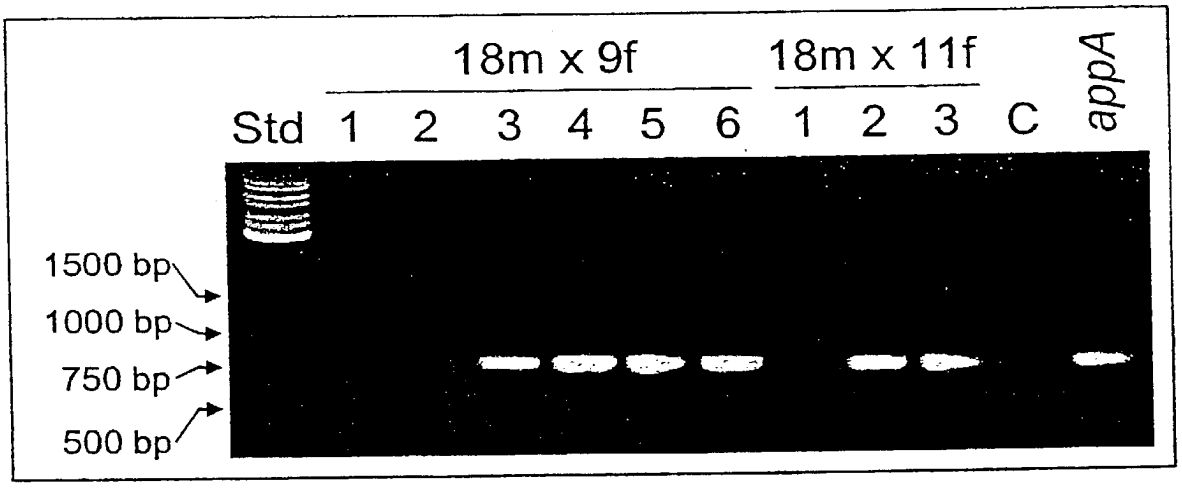

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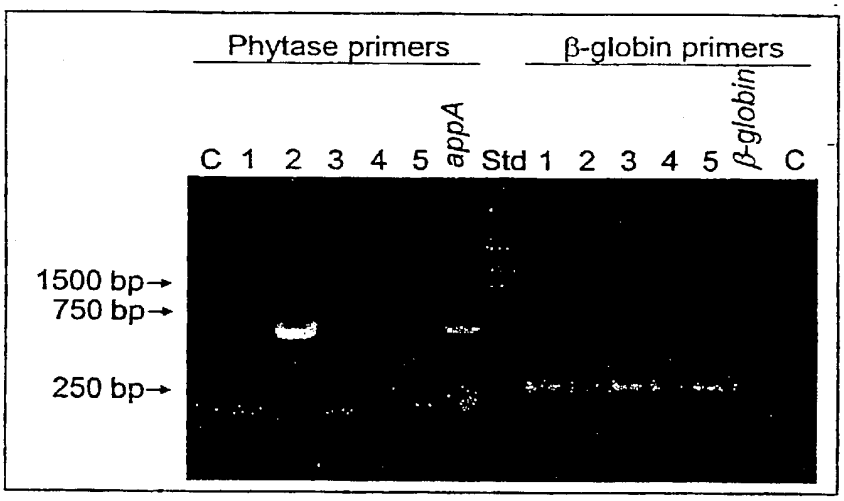
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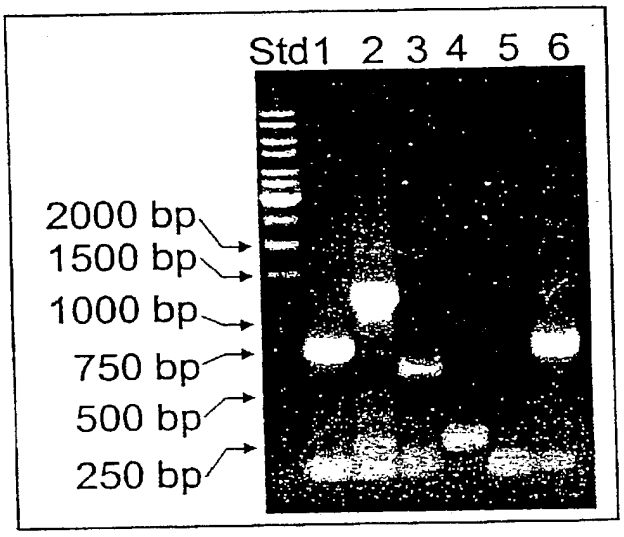
Figure 7



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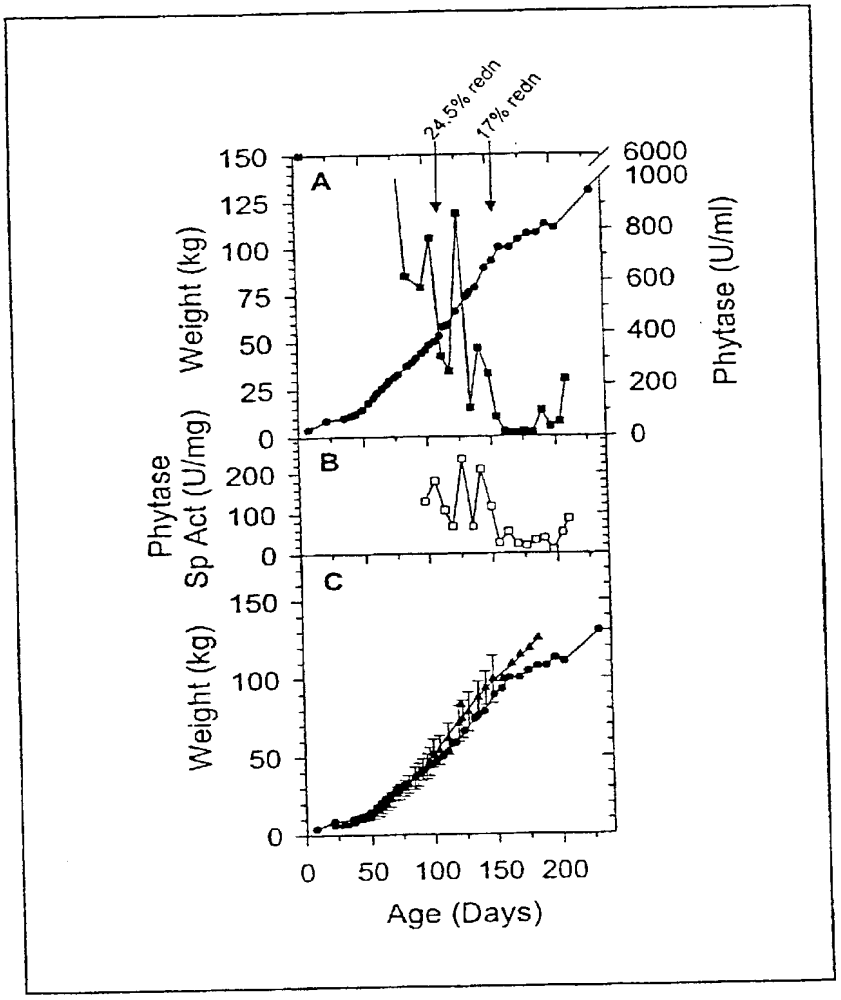
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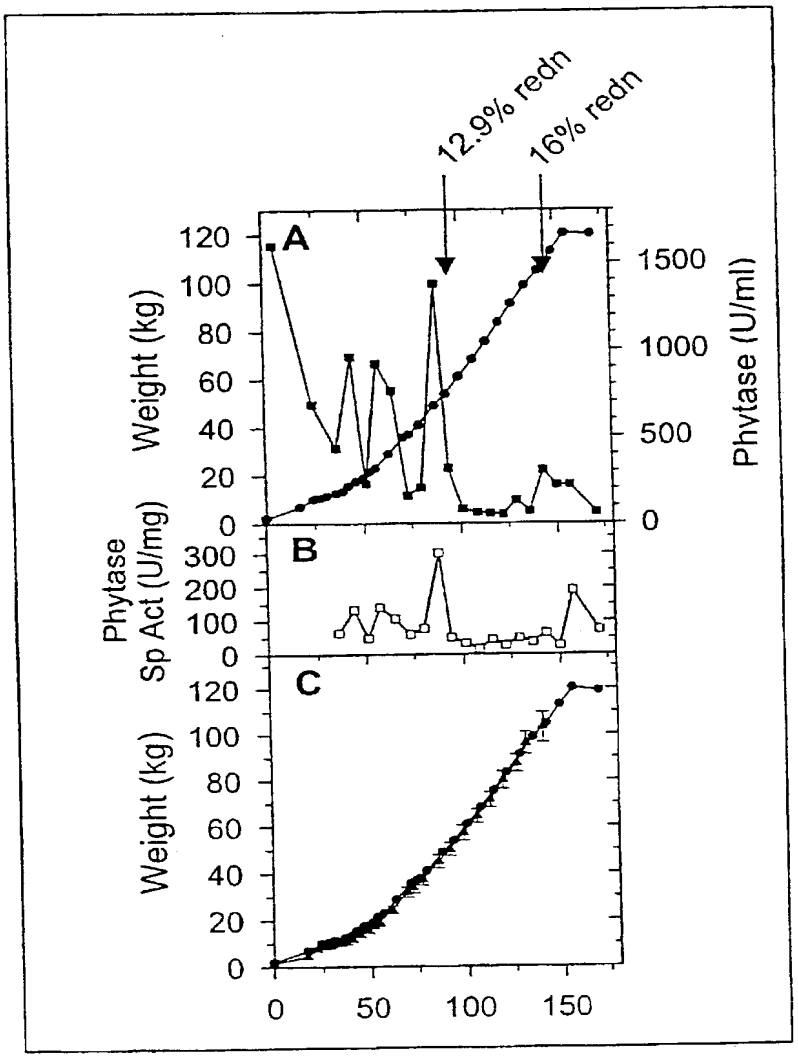
Figure 9



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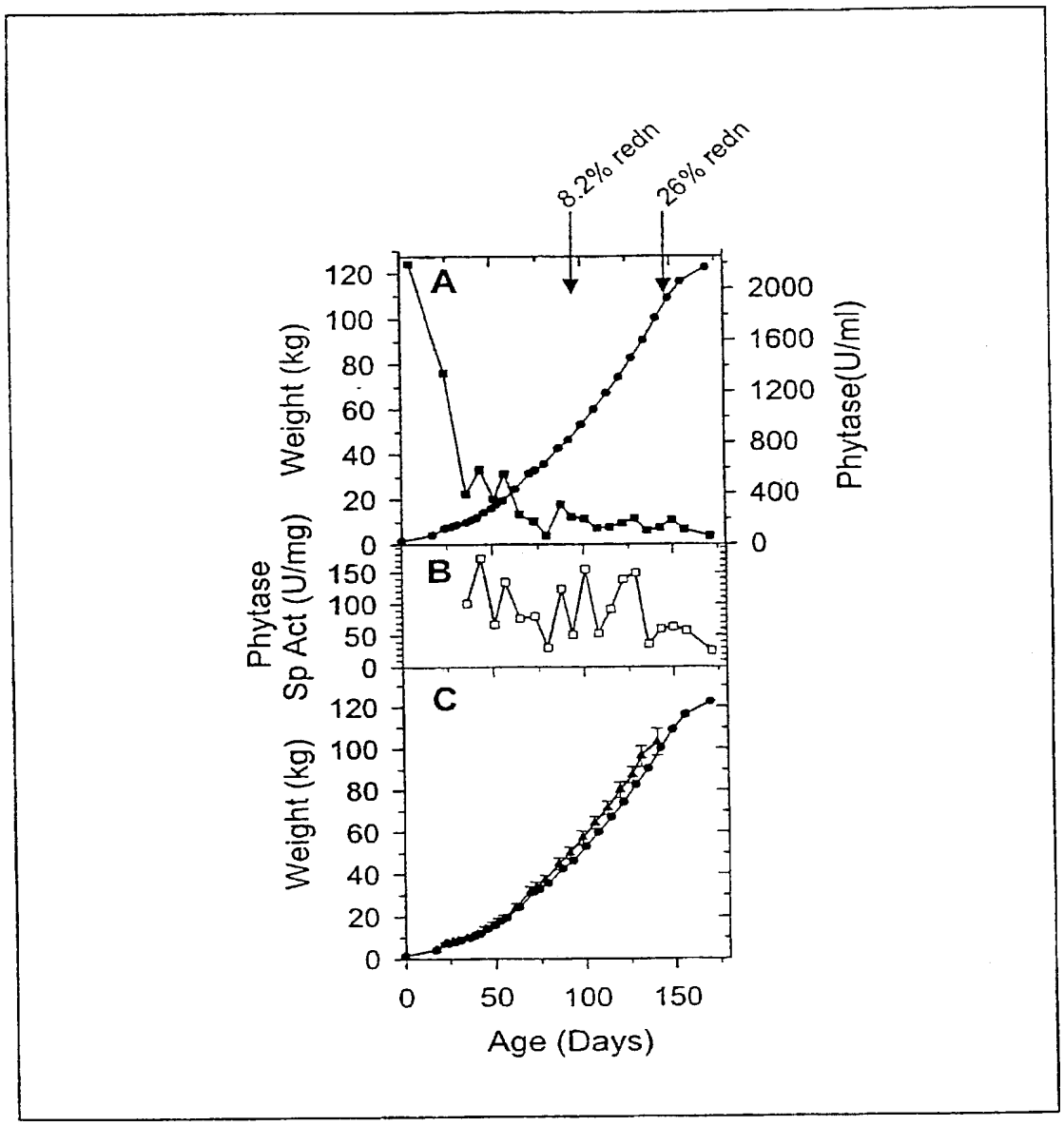
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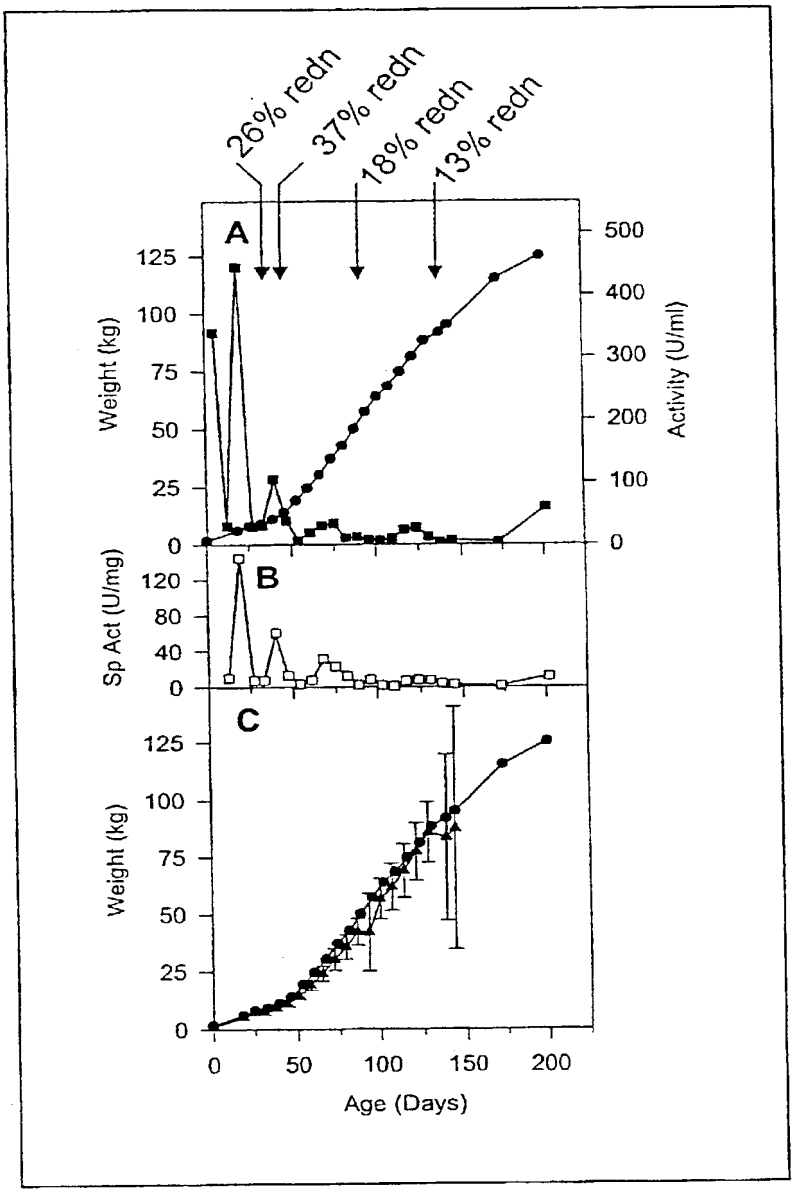
Figure 11



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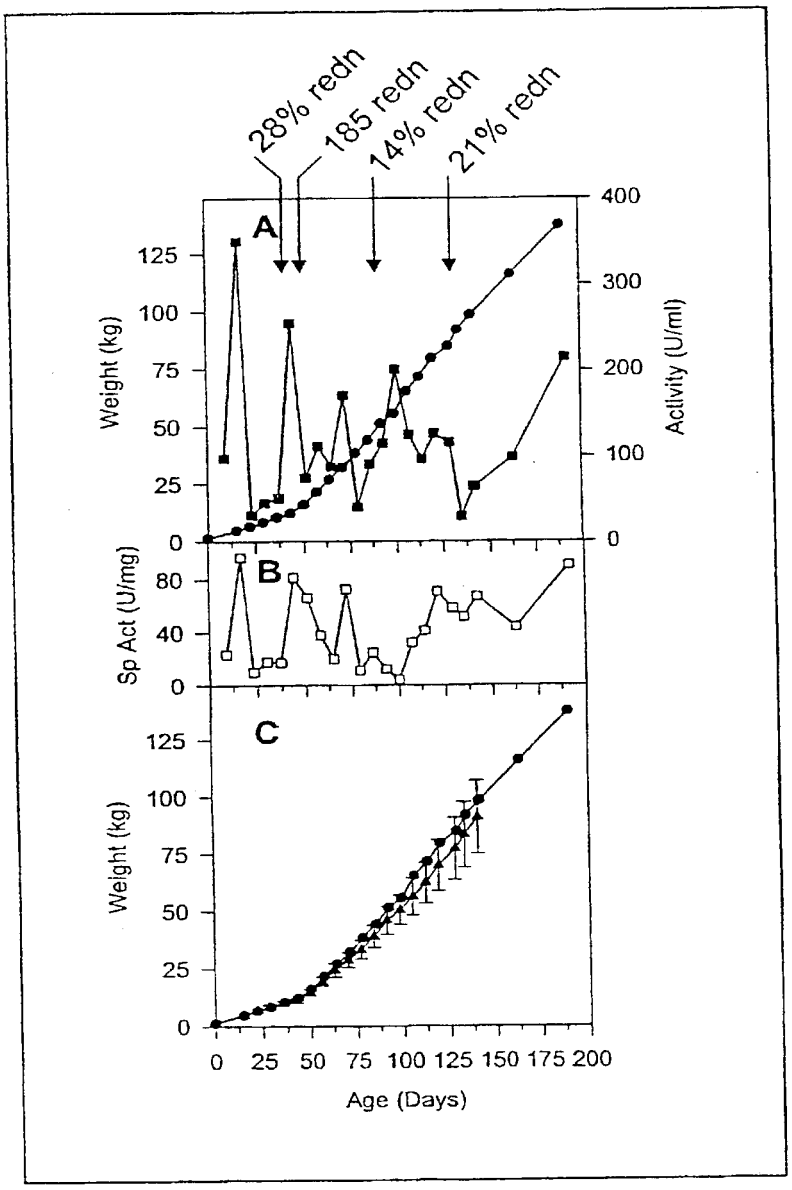

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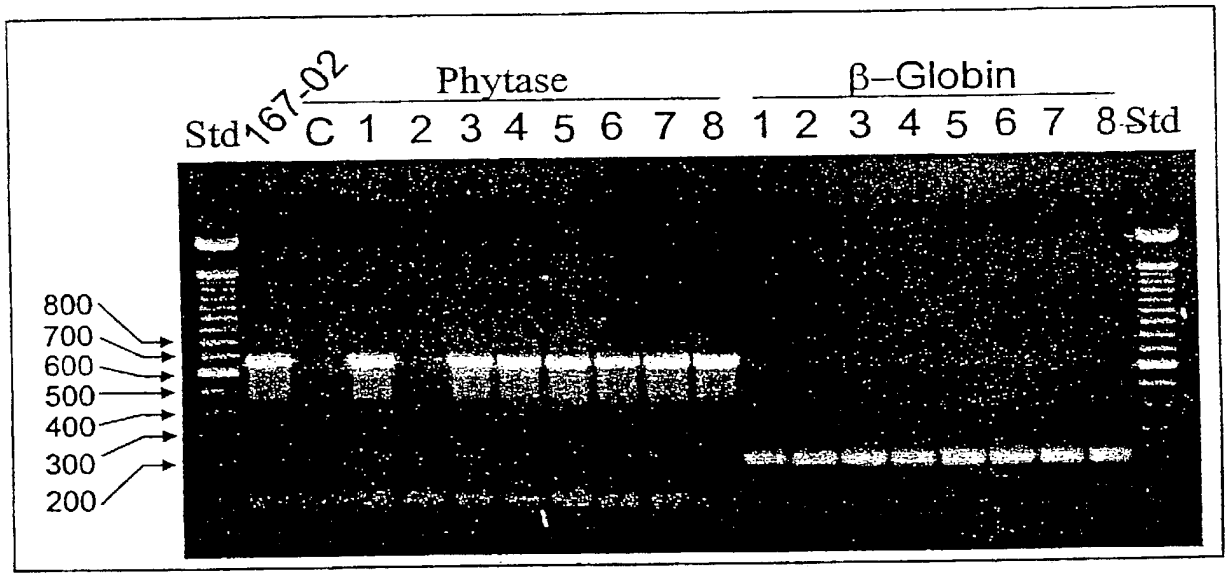

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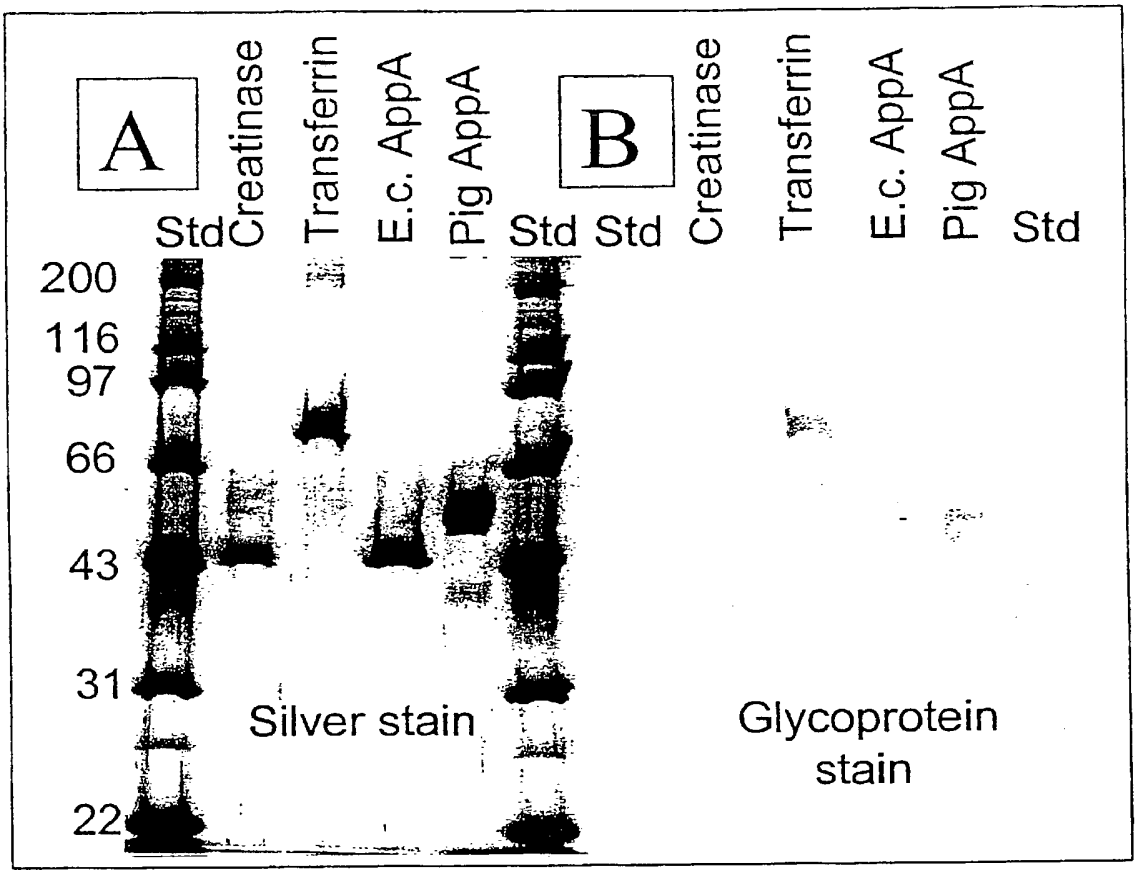
A
Std
Creatinase
Transferrin
E.c. AppA
Pig AppA
Std
200
116
97
66
43
31
22
Silver stain
B
Std
Creatinase
Transferrin
E.c. AppA
Pig AppA
Std
Glycoprotein stain

Figure 15

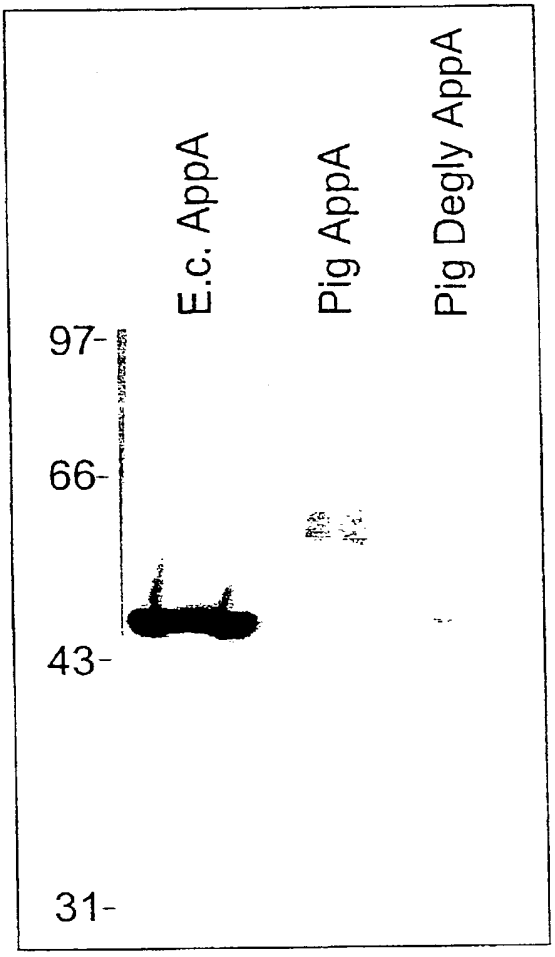

Figure 15B

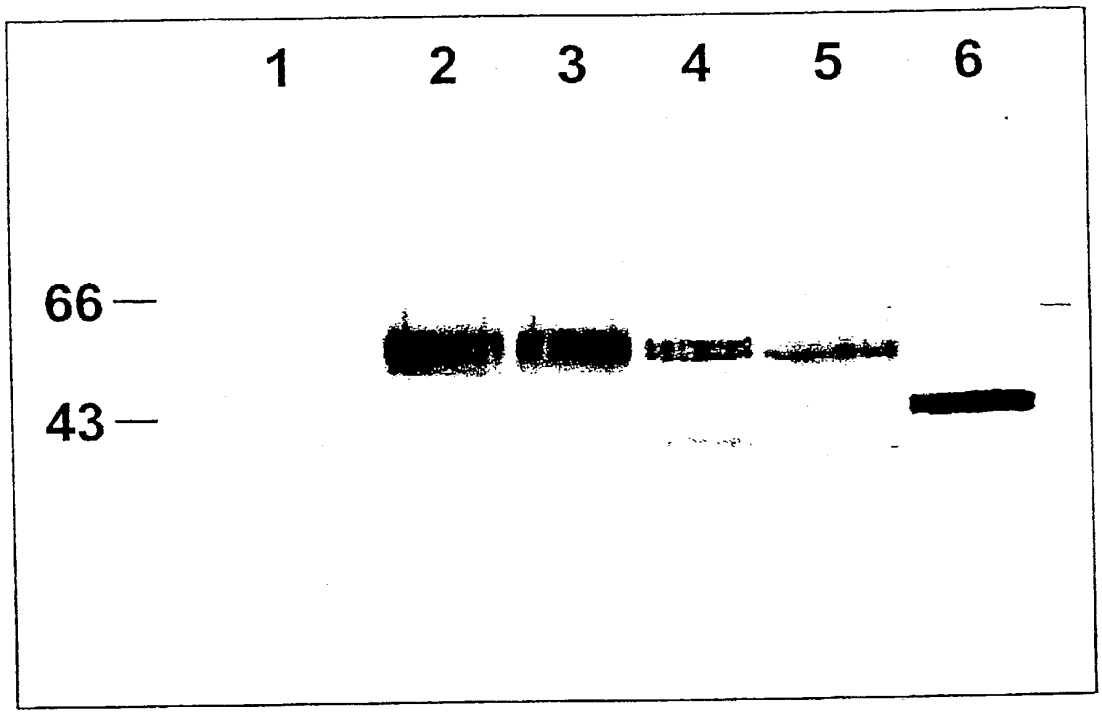

Figure 16

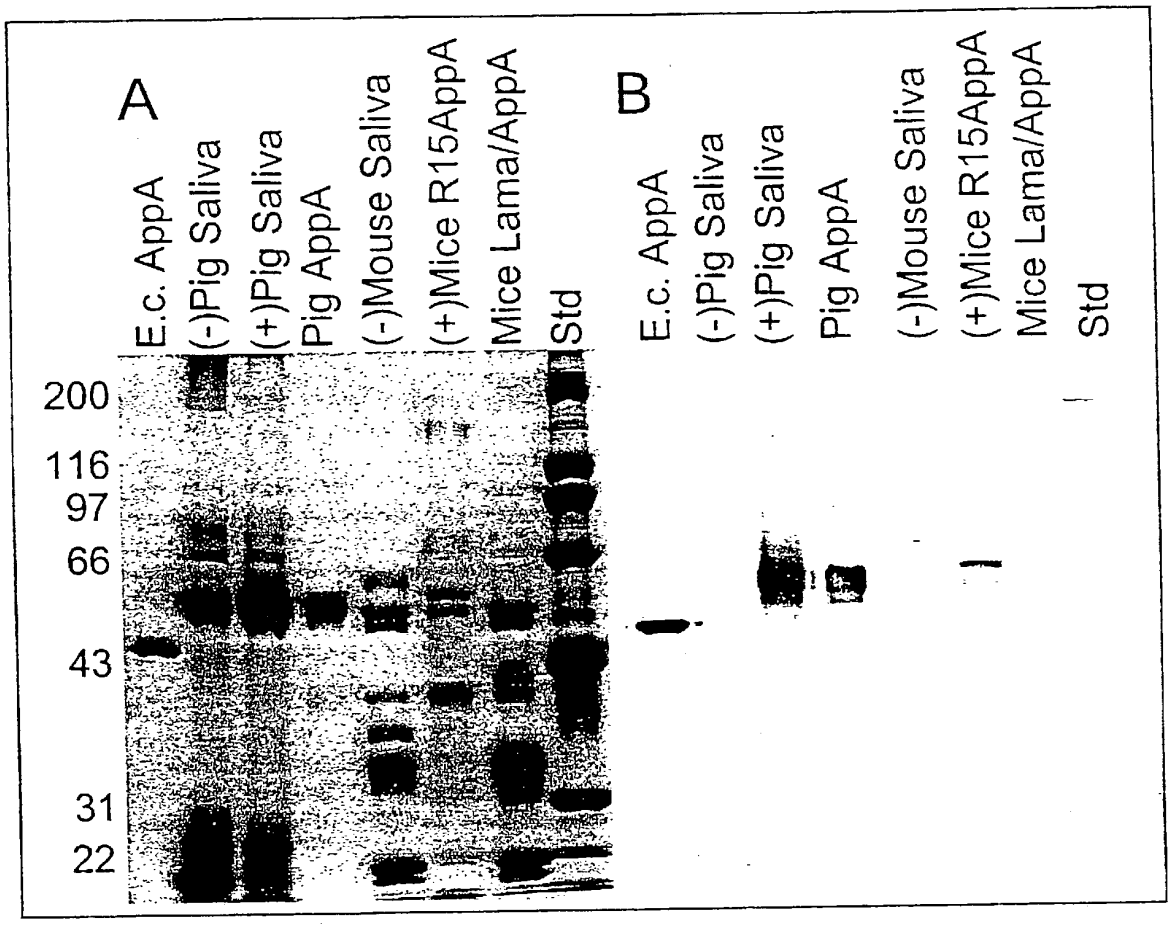

Figure 17

Figure 18: Nucleic acid sequence of the known segment of the R15/appa+intron plasmid, including the vector sequences of pBLCAT3 (SEQ ID NO:2).

```
LOCUS       R15/appa+intron    6708 bp    DNA     SYN       15-APR-2000
DEFINITION  R15/appa+intron transgene  with vector cut 13543 to 4954
ACCESSION R15/appa+intron
REFERENCE   1  (bases 1 to 6708))
SOURCE      synthetic construct.
      ORGANISM  synthetic construct
                artificial sequence.
KEYWORDS    salivary proline-rich protein, acid glucose-1-phosphatase; appA
            gene; periplasmic phosphoanhydride phosphohydrolase; artificial
            sequence;
AUTHORS     Golovan, S., Forsberg, C.W., Phillips, J.
  JOURNAL   Unpublished.

     DEFINITION  Rat salivary proline-rich protein (RP15) gene.
     ACCESSION   M64793 M36414
     VERSION     M64793.1  GI:206711
     SOURCE      Rat (Sprague-Dawley) liver DNA.
       ORGANISM  Rattus norvegicus
                 Eukaryota; Metazoa; Chordata; Craniata; Vertebrata;
Mammalia;
                 Eutheria; Rodentia; Sciurognathi; Muridae; Murinae;
Rattus.
     REFERENCE   1  (bases 1 to 1748)
       AUTHORS   Lin,H.H. and Ann,D.K.
       TITLE     Molecular characterization of rat multigene family
encoding
                 proline-rich proteins
       JOURNAL   Genomics 10, 102-113 (1991)
       MEDLINE   91257817
     FEATURES             Location/Qualifiers
          source          1..1748
                          /organism="Rattus norvegicus"
                          /strain="Sprague-Dawley"
                          /db_xref="taxon:10116"
                          /tissue_type="liver"
                          /tissue_lib="cosmid genomic library"
          misc_feature          1802-1810
                          /function=" consensus sequence for initiation in
                                    higher eukaryotes "


FEATURES             Location/Qualifiers
DEFINITION  E. coli periplasmic phosphoanhydride phosphohydrolase (appA)
gene,

     ACCESSION   M58708 L03370 L03371 L03372 L03373 L03374 L03375
     VERSION     M58708.1  GI:145283
     SOURCE            Escherichia coli DNA.
     ORGANISM    Escherichia coli
           Bacteria; Proteobacteria; gamma subdivision;
     Enterobacteriaceae;
           Escherichia.

REFERENCE   1   (bases 1811..3109)
     AUTHORS   Dassa,J., Marck,C. and Boquet,P.L.
```

Figure 18 (continued):

```
      TITLE     The complete nucleotide sequence of the Escherichia coli
                  gene appA reveals significant homology between pH 2.5
                  acid phosphatase and glucose-1-phosphatase
      JOURNAL   J. Bacteriol. 172 (9), 5497-5500 (1990)
      MEDLINE   90368616

FEATURES                Location/Qualifiers
      Source                  1811..3109
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                              /db_xref="taxon:562"
      sig_peptide             1811.. 1876
      /gene="appA"
      CDS                      1811..3109
                              /gene="appA"
                              /standard_name="acid phosphatase/phytase"
                              /transl_table=11
                              /product="periplasmic phosphoanhydride
                              phosphohydrolase"
                              /protein_id="AAA72086.1"
                              /db_xref="GI:145285"

/translation="MKAILIPFLSLLIPLTPQSAFAQSEPELKLESVVIVSRHGVRAP

TKATQLMQDVTPDAWPTWPVKLGWLTPRGGELIAYLGHYQRQRLVADGLLAKKGCPQS

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NVTDAILSRAGGSIADFTGHRQTAFRELERVLNFPQSNLCLKREKQDESCSLTQALPS

ELKVSADNVSLTGAVSLASMLTEIFLLQQAQGMPEPGWGRITDSHQWNTLLSLHNAQF

YLLQRTPEVARSRATPLLDLIKTALTPHPPQKQAYGVTLPTSVLFIAGHDTNLANLGG

ALELNWTLPGQPDNTPPGGELVFERWRRLSDNSQWIQVSLVFQTLQQMRDKTPLSLNT
                        PPGEVKLTLAGCEERNAQGMCSLAGFTQIVNEARIPACSL"
      mat_peptide       1877  3106
                        /gene="appA"
                        /product="periplasmic phosphoanhydride
                              phosphohydrolase"

      mutation    replace(1817.. 1819,"gcg changed to gcc")
                  /gene="appA"
                  /standard_name="A3 mutant"
                  /note="created by site directed mutagenesis"
                  /phenotype="silent mutation"
      mutation    replace(3092..3094," ccg changed to ccc")
                  /gene="appA"
                  /standard_name=" P428 mutant"
                  /note="created by site directed mutagenesis"
                  /phenotype=" silent mutation "
      mutation    replace(3095..3097," gcg changed to gct")
                  /gene="appA"
                  /standard_name=" A429 mutant"
                  /note="created by site directed mutagenesis"
                  /phenotype=" silent mutation "
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Figure 18 (continued):

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DEFINITION  Plasmid pBLCAT3  (bases 3109 to 6708)
     ACCESSION   X64409
     VERSION     X64409.1  GI:58163
     SOURCE      synthetic construct.
       ORGANISM  synthetic construct
                 artificial sequence.
     REFERENCE   1  (bases 3109 to 6708)
       AUTHORS   Luckow,B.H.R.
       TITLE     Direct Submission
       JOURNAL   Submitted (06-FEB-1992) B.H.R. Luckow, German Cancer Res
                 Center, Im Neuenheimer Feld 280, W-6900 Heidelberg, FRG
     REFERENCE   2  (bases 3109 to 6708)
       AUTHORS   Luckow,B. and Schutz,G.
       TITLE     CAT constructions with multiple unique restriction sites
for
                 the functional analysis of eukaryotic promoters and
regulatory
                 elements
       JOURNAL   Nucleic Acids Res. 15 (13), 5490 (1987)
       MEDLINE   87260024
     COMMENT     Promoterless CAT vector for transient transfection
experiments
                  with eukaryotic cells. Allows the analysis of foreign
                  promoters and  enhancers.
     FEATURES             Location/Qualifiers
          source          3109 to 6116
                          /organism="synthetic construct"
                          /db_xref="taxon:32630"

          SV40 t intron   3197..3810
                          /note="SV40 signals"
          polyA_signal    3807..4047
                          /note="SV40 signals"
          CDS             complement(5244..6104)
                          /codon_start=1
                          /transl_table=11
                          /gene="Amp"
                          /product="beta-lactamase"
                          /protein_id="CAA45753.1"
                          /db_xref="GI:58165"

 BASE COUNT     1916 a   1479 c   1515 g   1798 t
 ORIGIN
        1 GGATCCCCTT TGCTATGTAG TTTTTAATGG AAATTACAAC CCATAGTGTG TTGATAAATA
       61 GAGAGTCCTG TTTGGTTTAA GCAACCTCTG TTTCTCATAA ACTCCATAAA AACAGGAATA
      121 CTCTTTGTTT CTAGCATAAC CAAAAGATTT AGTGAATTGA AAACAATGTT CCCTTAGAGT
      181 ATAGGTCTAA TAACCCCGAA AATATTACCA TGATACTGAG CATTTGTAAG TATCTCATAG
      241 CATGTAGTAT CCATAGTCCA TCAATGAGAG AGACATTTAA CATGATTTTC ATTAATCAGG
      301 TGGAAAAGAC ATGACAACAT TCACAGGCAC TGCACAGAAC ATAGTGGTCC ACCTTGCACA
      361 TATTTCACTA AACTAGGTTT ATCTATTTTG TTGCTTTCTC TAACATCTCT GCAATGAAGC
      421 AGGTCAACAG TGCCACATAT CCTTTACTTA ACCTAAGGAA CACAAAAAAT TTTCTACATA
      481 TATCCTGGTT AGAGAGTGCT TAAAATAAGT TTTCCAAGAA TGGAAAAGAA ATGTTCTGAC
      541 TTAACAATTA AGACAGTATT TATTTAAAGC AAGAAATATG AGGCACACAA GAAAATATTT
      601 TGGGAAGAAA CCATTTGGTG AACAATATTT CAAATAAAAA TAGACAAACA TAGTTAATTG
      661 TAAAACATAT GTTTGACCAG CCCTTCTTTT CAATAGGCTT AATGTGAATA AAATGTTAAA
      721 GATTCTCTTT GGGTGGCTGC AAATTGTCCA CGAATAAGAC AAAATATAAA AATAAGGACT
      781 GAGTCTCACA AAATGAAAAG GAAATATATT CAGAAAGAGA ATCTTGAGAG AATGTGTTGT
      841 CACAAATTAA AGAAAACCTG TGGTGAATGA CATCCTGAGG CCTGAGCTAT TACTGACATT
```

Figure 18 (continued):

```
 901 TAAGATAAAG GTAACTGTAT ACATTTGTCC CATTGAGGGG ACAAGAAAGC TGCTCTCATG
 961 TTCAGCTCTA TAATTCTTGC CTTAAACAAC TTAAATAGAA TGATTTAAAA TATGGAGCTG
1021 TCCATGGACC TTTGAAATAT AAAATAGTCA AGCAACTTAT CAAGGAATTA CAGATTCCTT
1081 GATACTAACA CAGGTAAATC CCACACGTGT TTTGAGACTA CATTTGCTGG GATTTTATTG
1141 ATGTAATAGG TCACATGTTT TTCGGGCCAA TGTTGCTGTT ATTCGGTTAC TTCAAGAGAA
1201 TAGTGGCAAC TGATGCTATG TATTCTAGGG GTTTGAAGTG ATGTTTCATG ATTGAAATTT
1261 GTAAAAGAAT AACATCATCA TTCTTAACAA TAGAACATAT AAAGTCACAC AGAAGTGACA
1321 GTGTTTAAGC TGTACTATTG ATCAAAGAAA TTTATTACCT TCAGTTTCAA TGGAAATAAT
1381 TACTGATAAT ACAAACATGT GTGAACACAC ACTAATCCTA TCCAAATGCA CAGTGATACA
1441 CAGAAAATAT TAGCAAGTAG AATGCAATAT TTATATAACG ATTGTATTTA TCAATCAATT
1501 GTATGTATCA ATATATGGGC TATTTTCTTA CACATGATTT TATTCAAATT TACTCTAATC
1561 ATTGTTGAAC CATTTAGAAA AGGCATACTG GCAACTTTTC CTTACCTCAT CCAGCTGGGC
1621 AAAAGTCCCA GTGTGGAGTA AAGGATGCAA GATTTCCTGC TCTGTTAAGT ATAAAATAAT
1681 AGTATGAATT CAAAGGTGCC ATTCTTCTGC TTCTAGTTAT AAAGGCAGTG CTTGCTTCTT
1741 CCAGCACAGA TCTGGATCTC GAGGAGCTTG GCGAGATTTT CAGGAGCTAA GGAAGCTAAA
1801 AGCCGCCACC ATGAAAGCCA TCTTAATCCC ATTTTTATCT CTTCTGATTC CGTTAACCCC
1861 GCAATCTGCA TTCGCTCAGA GTGAGCCGGA GCTGAAGCTG GAAAGTGTGG TGATTGTCAG
1921 TCGTCATGGT GTGCGTGCTC CAACCAAGGC CACGCAACTG ATGCAGGATG TCACCCCAGA
1981 CGCATGGCCA ACCTGGCCGG TAAAACTGGG TTGGCTGACA CCGCGCGGTG GTGAGCTAAT
2041 CGCCTATCTC GGACATTACC AACGCCAGCG TCTGGTAGCC GACGGATTGC TGGCGAAAAA
2101 GGGCTGCCCG CAGTCTGGTC AGGTCGCGAT TATTGCTGAT GTCGACGAGC GTACCCGTAA
2161 AACAGGCGAA GCCTTCGCCG CCGGGCTGGC ACCTGACTGT GCAATAACCG TACATACCCA
2221 GGCAGATACG TCCAGTCCCG ATCCGTTATT TAATCCTCTA AAAACTGGCG TTTGCCAACT
2281 GGATAACGCG AACGTGACTG ACGCGATCCT CAGCAGGGCA GGAGGGTCAA TTGCTGACTT
2341 TACCGGGCAT CGGCAAACGG CGTTTCGCGA ACTGGAACGG GTGCTTAATT TTCCGCAATC
2401 AAACTTGTGC CTTAAACGTG AGAAACAGGA CGAAAGCTGT TCATTAACGC AGGCATTACC
2461 ATCGGAACTC AAGGTGAGCG CCGACAATGT CTCATTAACC GGTGCGGTAA GCCTCGCATC
2521 AATGCTGACG GAGATATTTC TCCTGCAACA AGCACAGGGA ATGCCGGAGC CGGGGTGGGG
2581 AAGGATCACC GATTCACACC AGTGGAACAC CTTGCTAAGT TTGCATAACG CGCAATTTTA
2641 TTTGCTACAA CGCACGCCAG AGGTTGCCCG CAGCCGCGCC ACCCCGTTAT TAGATTTGAT
2701 CAAGACAGCG TTGACGCCCC ATCCACCGCA AAAACAGGCG TATGGTGTGA CATTACCCAC
2761 TTCAGTGCTG TTTATCGCCG GACACGATAC TAATCTGGCA AATCTCGGCG GCGCACTGGA
2821 GCTCAACTGG ACGCTTCCCG GTCAGCCGGA TAACACGCCG CCAGGTGGTG AACTGGTGTT
2881 TGAACGCTGG CGTCGGCTAA GCGATAACAG CCAGTGGATT CAGGTTTCGC TGGTCTTCCA
2941 GACTTTACAG CAGATGCGTG ATAAAACGCC GCTGTCATTA AATACGCCGC CCGGAGAGGT
3001 GAAACTGACC CTGGCAGGAT GTGAAGAGCG AAATGCGCAG GGCATGTGTT CGTTGGCAGG
3061 TTTTACGCAA ATCGTGAATG AAGCACGCAT ACCCGCTTGC AGTTTGTAAG GTATAAGGCA
3121 GTTATTGGTG CCCTTAAACG CCTGGTGCTA CGCCTGAATA AGTGATAATA AGCGGATGAA
3181 TGGCAGAAAT TCGCCGGATC TTTGTGAAGG AACCTTACTT CTGTGGTGTG ACATAATTGG
3241 ACAAACTACC TACAGAGATT TAAAGCTCTA AGGTAAATAT AAAATTTTTA AGTGTATAAT
3301 GTGTTAAACT ACTGATTCTA ATTGTTTGTG TATTTTAGAT TCCAACCTAT GGAACTGATG
3361 AATGGGAGCA GTGGTGGAAT GCCTTTAATG AGGAAAACCT GTTTTGCTCA GAAGAAATGC
3421 CATCTAGTGA TGATGAGGCT ACTGCTGACT CTCAACATTC TACTCCTCCA AAAAAGAAGA
3481 GAAAGGTAGA AGACCCCAAG GACTTTCCTT CAGAATTGCT AAGTTTTTTG AGTCATGCTG
3541 TGTTTAGTAA TAGAACTCTT GCTTGCTTTG CTATTTACAC CACAAAGGAA AAAGCTGCAC
3601 TGCTATACAA GAAAATTATG GAAAAATATT CTGTAACCTT TATAAGTAGG CATAACAGTT
3661 ATAATCATAA CATACTGTTT TTTCTTACTC CACACAGGCA TAGAGTGTCT GCTATTAATA
3721 ACTATGCTCA AAAATTGTGT ACCTTTAGCT TTTTAATTTG TAAAGGGGTT AATAAGGAAT
3781 ATTTGATGTA TAGTGCCTTG ACTAGAGATC ATAATCAGCC ATACCACATT TGTAGAGGTT
3841 TTACTTGCTT TAAAAAACCT CCCACACCTC CCCCTGAACC TGAAACATAA AATGAATGCA
3901 ATTGTTGTTG TTAACTTGTT TATTGCAGCT TATAATGGTT ACAAATAAAG CAATAGCATC
3961 ACAAATTTCA CAAATAAAGC ATTTTTTTCA CTGCATTCTA GTTGTGGTTT GTCCAAACTC
4021 ATCAATGTAT CTTATCATGT CTGGATCGAT CCCCGGGTAC CGAGCTCGAA TTCGTAATCA
4081 TGGTCATAGC TGTTTCCTGT GTGAAATTGT TATCCGCTCA CAATTCCACA CAACATACGA
4141 GCCGGAAGCA TAAAGTGTAA AGCCTGGGGT GCCTAATGAG TGAGCTAACT CACATTAATT
4201 GCGTTGCGCT CACTGCCCGC TTTCCAGTCG GGAAACCTGT CGTGCCAGCT GCATTAATGA
4261 ATCGGCCAAC GCGCGGGGAG AGGCGGTTTG CGTATTGGGC GCTCTTCCGC TTCCTCGCTC
4321 ACTGACTCGC TGCGCTCGGT CGTTCGGCTG CGGCGAGCGG TATCAGCTCA CTCAAAGGCG
```

Figure 18 (continued):

```
4381 GTAATACGGT TATCCACAGA ATCAGGGGAT AACGCAGGAA AGAACATGTG AGCAAAAGGC
4441 CAGCAAAAGG CCAGGAACCG TAAAAAGGCC GCGTTGCTGG CGTTTTTCCA TAGGCTCCGC
4501 CCCCCTGACG AGCATCACAA AAATCGACGC TCAAGTCAGA GGTGGCGAAA CCCGACAGGA
4561 CTATAAAGAT ACCAGGCGTT TCCCCCTGGA AGCTCCCTCG TGCGCTCTCC TGTTCCGACC
4621 CTGCCGCTTA CCGGATACCT GTCCGCCTTT CTCCCTTCGG GAAGCGTGGC GCTTTCTCAA
4681 TGCTCACGCT GTAGGTATCT CAGTTCGGTG TAGGTCGTTC GCTCCAAGCT GGGCTGTGTG
4741 CACGAACCCC CCGTTCAGCC CGACCGCTGC GCCTTATCCG GTAACTATCG TCTTGAGTCC
4801 AACCCGGTAA GACACGACTT ATCGCCACTG GCAGCAGCCA CTGGTAACAG GATTAGCAGA
4861 GCGAGGTATG TAGGCGGTGC TACAGAGTTC TTGAAGTGGT GGCCTAACTA CGGCTACACT
4921 AGAAGGACAG TATTTGGTAT CTGCGCTCTG CTGAAGCCAG TTACCTTCGG AAAAAGAGTT
4981 GGTAGCTCTT GATCCGGCAA ACAAACCACC GCTGGTAGCG GTGGTTTTTT TGTTTGCAAG
5041 CAGCAGATTA CGCGCAGAAA AAAAGGATCT CAAGAAGATC CTTTGATCTT TTCTACGGGG
5101 TCTGACGCTC AGTGGAACGA AAACTCACGT TAAGGGATTT TGGTCATGAG ATTATCAAAA
5161 AGGATCTTCA CCTAGATCCT TTTAAATTAA AAATGAAGTT TTAAATCAAT CTAAAGTATA
5221 TATGAGTAAA CTTGGTCTGA CAGTTACCAA TGCTTAATCA GTGAGGCACC TATCTCAGCG
5281 ATCTGTCTAT TTCGTTCATC CATAGTTGCC TGACTCCCCG TCGTGTAGAT AACTACGATA
5341 CGGGAGGGCT TACCATCTGG CCCCAGTGCT GCAATGATAC CGCGAGACCC ACGCTCACCG
5401 GCTCCAGATT TATCAGCAAT AAACCAGCCA GCCGGAAGGG CCGAGCGCAG AAGTGGTCCT
5461 GCAACTTTAT CCGCCTCCAT CCAGTCTATT AATTGTTGCC GGGAAGCTAG AGTAAGTAGT
5521 TCGCCAGTTA ATAGTTTGCG CAACGTTGTT GCCATTGCTA CAGGCATCGT GGTGTCACGC
5581 TCGTCGTTTG GTATGGCTTC ATTCAGCTCC GGTTCCCAAC GATCAAGGCG AGTTACATGA
5641 TCCCCCATGT TGTGCAAAAA AGCGGTTAGC TCCTTCGGTC CTCCGATCGT TGTCAGAAGT
5701 AAGTTGGCCG CAGTGTTATC ACTCATGGTT ATGGCAGCAC TGCATAATTC TCTTACTGTC
5761 ATGCCATCCG TAAGATGCTT TTCTGTGACT GGTGAGTACT CAACCAAGTC ATTCTGAGAA
5821 TAGTGTATGC GGCGACCGAG TTGCTCTTGC CCGGCGTCAA TACGGGATAA TACCGCGCCA
5881 CATAGCAGAA CTTTAAAAGT GCTCATCATT GGAAAACGTT CTTCGGGGCG AAAACTCTCA
5941 AGGATCTTAC CGCTGTTGAG ATCCAGTTCG ATGTAACCCA CTCGTGCACC CAACTGATCT
6001 TCAGCATCTT TTACTTTCAC CAGCGTTTCT GGGTGAGCAA AAACAGGAAG GCAAAATGCC
6061 GCAAAAAAGG GAATAAGGGC GACACGGAAA TGTTGAATAC TCATACTCTT CCTTTTTCAA
6121 TATTATTGAA GCATTTATCA GGGTTATTGT CTCATGAGCG GATACATATT TGAATGTATT
6181 TAGAAAAATA AACAAATAGG GGTTCCGCGC ACATTTCCCC GAAAAGTGCC ACCTGACGTC
6241 TAAGAAACCA TTATTATCAT GACATTAACC TATAAAAATA GGCGTATCAC GAGGCCCTTT
6301 CGTCTCGCGC GTTTCGGTGA TGACGGTGAA AACCTCTGAC ACATGCAGCT CCCGGAGACG
6361 GTCACAGCTT GTCTGTAAGC GGATGCCGGG AGCAGACAAG CCCGTCAGGG CGCGTCAGCG
6421 GGTGTTGGCG GGTGTCGGGG CTGGCTTAAC TATGCGGCAT CAGAGCAGAT TGTACTGAGA
6481 GTGCACCATA TGCGGTGTGA AATACCGCAC AGATGCGTAA GGAGAAAATA CCGCATCAGG
6541 CGCCATTCGC CATTCAGGCT GCGCAACTGT TGGGAAGGGC GATCGGTGCG GGCCTCTTCG
6601 CTATTACGCC AGCTGGCGAA AGGGGGATGT GCTGCAAGGC GATTAAGTTG GGTAACGCCA
6661 GGGTTTTCCC AGTCACGACG TTGTAAAACG ACGGCCAGTG CCAAGCTT
```

//

Figure 19: Nucleic acid sequence of the known segment of the R15/appa+intron transgene used for the generation of transgenic mice (SEQ ID NO: 3).

```
LOCUS       R15/appa      4060 bp    DNA          SYN        15-APR-2000
DEFINITION  R15/appa transgene  without vector
ACCESSION R15/appa
REFERENCE   1  (bases 1 to 4060)
SOURCE      synthetic construct.
      ORGANISM  synthetic construct
                artificial sequence.
KEYWORDS    salivary proline-rich protein, acid glucose-1-phosphatase; appA
            gene; periplasmic phosphoanhydride phosphohydrolase; artificial
            sequence;
AUTHORS     Golovan, S., Forsberg, C.W., Phillips, J.
  JOURNAL   Unpublished.

     DEFINITION  Rat salivary proline-rich protein (RP15) gene.
     ACCESSION   M64793 M36414
     VERSION     M64793.1  GI:206711
     SOURCE      Rat (Sprague-Dawley) liver DNA.
       ORGANISM  Rattus norvegicus
                 Eukaryota; Metazoa; Chordata; Craniata; Vertebrata;
Mammalia;
                 Eutheria; Rodentia; Sciurognathi; Muridae; Murinae;
Rattus.
     REFERENCE   1  (bases 1 to 1748)
       AUTHORS   Lin,H.H. and Ann,D.K.
       TITLE     Molecular characterization of rat multigene family
encoding
                 proline-rich proteins
       JOURNAL   Genomics 10, 102-113 (1991)
       MEDLINE   91257817
     FEATURES             Location/Qualifiers
          source          1..1748
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                          /strain="Sprague-Dawley"
                          /db_xref="taxon:10116"
                          /tissue_type="liver"
                          /tissue_lib="cosmid genomic library"
          misc_feature          1802-1810
                          /function=" consensus sequence for initiation in
                                     higher eukaryotes "


FEATURES             Location/Qualifiers
DEFINITION  E. coli periplasmic phosphoanhydride phosphohydrolase (appA)
gene,

      ACCESSION   M58708 L03370 L03371 L03372 L03373 L03374 L03375
      VERSION     M58708.1  GI:145283
      SOURCE            Escherichia coli DNA.
      ORGANISM    Escherichia coli
            Bacteria; Proteobacteria; gamma subdivision;
      Enterobacteriaceae;
            Escherichia.

REFERENCE   1   (bases 1811..3109)
      AUTHORS   Dassa,J., Marck,C. and Boquet,P.L.
```

Figure 19 (continued):

```
      TITLE     The complete nucleotide sequence of the Escherichia coli
                  gene appA reveals significant homology between pH 2.5
                  acid phosphatase and glucose-1-phosphatase
      JOURNAL   J. Bacteriol. 172 (9), 5497-5500 (1990)
      MEDLINE   90368616

FEATURES                Location/Qualifiers
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      /gene="appA"
CDS                   1811..3109
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                              /standard_name="acid phosphatase/phytase"
                              /transl_table=11
                              /product="periplasmic phosphoanhydride
                              phosphohydrolase"
                              /protein_id="AAA72086.1"
                              /db_xref="GI:145285"

/translation="MKAILIPFLSLLIPLTPQSAFAQSEPELKLESVVIVSRHGVRAP

TKATQLMQDVTPDAWPTWPVKLGWLTPRGGELIAYLGHYQRQRLVADGLLAKKGCPQS

GQVAIIADVDERTRKTGEAFAAGLAPDCAITVHTQADTSSPDPLFNPLKTGVCQLDNA

NVTDAILSRAGGSIADFTGHRQTAFRELERVLNFPQSNLCLKREKQDESCSLTQALPS

ELKVSADNVSLTGAVSLASMLTEIFLLQQAQGMPEPGWGRITDSHQWNTLLSLHNAQF

YLLQRTPEVARSRATPLLDLIKTALTPHPPQKQAYGVTLPTSVLFIAGHDTNLANLGG

ALELNWTLPGQPDNTPPGGELVFERWRRLSDNSQWIQVSLVFQTLQQMRDKTPLSLNT
                              PPGEVKLTLAGCEERNAQGMCSLAGFTQIVNEARIPACSL"
      mat_peptide             1877  3106
                              /gene="appA"
                              /product="periplasmic phosphoanhydride
                                    phosphohydrolase"

      mutation          replace(1817.. 1819,"gcg changed to gcc")
                        /gene="appA"
                        /standard_name="A3 mutant"
                        /note="created by site directed mutagenesis"
                        /phenotype="silent mutation"
      mutation          replace(3092..3094," ccg changed to ccc")
                        /gene="appA"
                        /standard_name=" P428 mutant"
                        /note="created by site directed mutagenesis"
                        /phenotype=" silent mutation "
      mutation          replace(3095..3097," gcg changed to gct")
                        /gene="appA"
                        /standard_name=" A429 mutant"
                        /note="created by site directed mutagenesis"
                        /phenotype=" silent mutation "
```

Figure 19 (continued):

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     SV40 t intron    3197..3810
                      /note="SV40 signals"
     polyA_signal     3807..4047
                      /note="SV40 signals"

BASE COUNT     1257 a    814 c    843 g   1146 t
ORIGIN
        1 GGATCCCCTT TGCTATGTAG TTTTTAATGG AAATTACAAC CCATAGTGTG TTGATAAATA
       61 GAGAGTCCTG TTTGGTTTAA GCAACCTCTG TTTCTCATAA ACTCCATAAA AACAGGAATA
      121 CTCTTTGTTT CTAGCATAAC CAAAAGATTT AGTGAATTGA AAACAATGTT CCCTTAGAGT
      181 ATAGGTCTAA TAACCCCGAA AATATTACCA TGATACTGAG CATTTGTAAG TATCTCATAG
      241 CATGTAGTAT CCATAGTCCA TCAATGAGAG AGACATTTAA CATGATTTTC ATTAATCAGG
      301 TGGAAAAGAC ATGACAACAT TCACAGGCAC TGCACAGAAC ATAGTGGTCC ACCTTGCACA
      361 TATTTCACTA AACTAGGTTT ATCTATTTTG TTGCTTTCTC TAACATCTCT GCAATGAAGC
      421 AGGTCAACAG TGCCACATAT CCTTTACTTA ACCTAAGGAA CACAAAAAAT TTTCTACATA
      481 TATCCTGGTT AGAGAGTGCT TAAAATAAGT TTTCCAAGAA TGGAAAAGAA ATGTTCTGAC
      541 TTAACAATTA AGACAGTATT TATTTAAAGC AAGAAATATG AGGCACACAA GAAAATATTT
      601 TGGGAAGAAA CCATTTGGTG AACAATATTT CAAATAAAAA TAGACAAACA TAGTTAATTG
      661 TAAAACATAT GTTTGACCAG CCCTTCTTTT CAATAGGCTT AATGTGAATA AAATGTTAAA
      721 GATTCTCTTT GGGTGGCTGC AAATTGTCCA CGAATAAGAC AAAATATAAA AATAAGGACT
      781 GAGTCTCACA AAATGAAAAG GAAATATATT CAGAAAGAGA ATCTTGAGAG AATGTGTTGT
      841 CACAAATTAA AGAAAACCTG TGGTGAATGA CATCCTGAGG CCTGAGCTAT TACTGACATT
      901 TAAGATAAAG GTAACTGTAT ACATTTGTCC CATTGAGGGG ACAAGAAAGC TGCTCTCATG
      961 TTCAGCTCTA TAATTCTTGC CTTAAACAAC TTAAATAGAA TGATTTAAAA TATGGAGCTG
     1021 TCCATGGACC TTTGAAATAT AAAATAGTCA AGCAACTTAT CAAGGAATTA CAGATTCCTT
     1081 GATACTAACA CAGGTAAATC CCACACGTGT TTTGAGACTA CATTTGCTGG GATTTTATTG
     1141 ATGTAATAGG TCACATGTTT TTCGGGCCAA TGTTGCTGTT ATTCGGTTAC TTCAAGAGAA
     1201 TAGTGGCAAC TGATGCTATG TATTCTAGGG GTTTGAAGTG ATGTTTCATG ATTGAAATTT
     1261 GTAAAAGAAT AACATCATCA TTCTTAACAA TAGAACATAT AAAGTCACAC AGAAGTGACA
     1321 GTGTTTAAGC TGTACTATTG ATCAAAGAAA TTTATTACCT TCAGTTTCAA TGGAAATAAT
     1381 TACTGATAAT ACAAACATGT GTGAACACAC ACTAATCCTA TCCAAATGCA CAGTGATACA
     1441 CAGAAAATAT TAGCAAGTAG AATGCAATAT TTATATAACG ATTGTATTTA TCAATCAATT
     1501 GTATGTATCA ATATATGGGC TATTTTCTTA CACATGATTT TATTCAAATT TACTCTAATC
     1561 ATTGTTGAAC CATTTAGAAA AGGCATACTG GCAACTTTTC CTTACCTCAT CCAGCTGGGC
     1621 AAAAGTCCCA GTGTGGAGTA AAGGATGCAA GATTTCCTGC TCTGTTAAGT ATAAAATAAT
     1681 AGTATGAATT CAAAGGTGCC ATTCTTCTGC TTCTAGTTAT AAAGGCAGTG CTTGCTTCTT
     1741 CCAGCACAGA TCTGGATCTC GAGGAGCTTG GCGAGATTTT CAGGAGCTAA GGAAGCTAAA
     1801 AGCCGCCACC ATGAAAGCCA TCTTAATCCC ATTTTTATCT CTTCTGATTC CGTTAACCCC
     1861 GCAATCTGCA TTCGCTCAGA GTGAGCCGGA GCTGAAGCTG GAAAGTGTGG TGATTGTCAG
     1921 TCGTCATGGT GTGCGTGCTC CAACCAAGGC CACGCAACTG ATGCAGGATG TCACCCCAGA
     1981 CGCATGGCCA ACCTGGCCGG TAAAACTGGG TTGGCTGACA CCGCGCGGTG GTGAGCTAAT
     2041 CGCCTATCTC GGACATTACC AACGCCAGCG TCTGGTAGCC GACGGATTGC TGGCGAAAAA
     2101 GGGCTGCCCG CAGTCTGGTC AGGTCGCGAT TATTGCTGAT GTCGACGAGC GTACCCGTAA
     2161 AACAGGCGAA GCCTTCGCCG CCGGGCTGGC ACCTGACTGT GCAATAACCG TACATACCCA
     2221 GGCAGATACG TCCAGTCCCG ATCCGTTATT TAATCCTCTA AAAACTGGCG TTTGCCAACT
     2281 GGATAACGCG AACGTGACTG ACGCGATCCT CAGCAGGGCA GGAGGGTCAA TTGCTGACTT
     2341 TACCGGGCAT CGGCAAACGG CGTTTCGCGA ACTGGAACGG GTGCTTAATT TTCCGCAATC
     2401 AAACTTGTGC CTTAAACGTG AGAAACAGGA CGAAAGCTGT TCATTAACGC AGGCATTACC
     2461 ATCGGAACTC AAGGTGAGCG CCGACAATGT CTCATTAACC GGTGCGGTAA GCCTCGCATC
     2521 AATGCTGACG GAGATATTTC TCCTGCAACA AGCACAGGGA ATGCCGGAGC CGGGGTGGGG
     2581 AAGGATCACC GATTCACACC AGTGGAACAC CTTGCTAAGT TTGCATAACG CGCAATTTTA
     2641 TTTGCTACAA CGCACGCCAG AGGTTGCCCG CAGCCGCGCC ACCCCGTTAT TAGATTTGAT
     2701 CAAGACAGCG TTGACGCCCC ATCCACCGCA AAAACAGGCG TATGGTGTGA CATTACCCAC
     2761 TTCAGTGCTG TTTATCGCCG GACACGATAC TAATCTGGCA AATCTCGGCG GCGCACTGGA
     2821 GCTCAACTGG ACGCTTCCCG GTCAGCCGGA TAACACGCCG CCAGGTGGTG AACTGGTGTT
     2881 TGAACGCTGG CGTCGGCTAA GCGATAACAG CCAGTGGATT CAGGTTTCGC TGGTCTTCCA
```

Figure 19 (continued):

```
2941 GACTTTACAG CAGATGCGTG ATAAAACGCC GCTGTCATTA AATACGCCGC CCGGAGAGGT
3001 GAAACTGACC CTGGCAGGAT GTGAAGAGCG AAATGCGCAG GGCATGTGTT CGTTGGCAGG
3061 TTTTACGCAA ATCGTGAATG AAGCACGCAT ACCCGCTTGC AGTTTGTAAG GTATAAGGCA
3121 GTTATTGGTG CCCTTAAACG CCTGGTGCTA CGCCTGAATA AGTGATAATA AGCGGATGAA
3181 TGGCAGAAAT TCGCCGGATC TTTGTGAAGG AACCTTACTT CTGTGGTGTG ACATAATTGG
3241 ACAAACTACC TACAGAGATT TAAAGCTCTA AGGTAAATAT AAAATTTTTA AGTGTATAAT
3301 GTGTTAAACT ACTGATTCTA ATTGTTTGTG TATTTTAGAT TCCAACCTAT GGAACTGATG
3361 AATGGGAGCA GTGGTGGAAT GCCTTTAATG AGGAAAACCT GTTTTGCTCA GAAGAAATGC
3421 CATCTAGTGA TGATGAGGCT ACTGCTGACT CTCAACATTC TACTCCTCCA AAAAAGAAGA
3481 GAAAGGTAGA AGACCCCAAG GACTTTCCTT CAGAATTGCT AAGTTTTTTG AGTCATGCTG
3541 TGTTTAGTAA TAGAACTCTT GCTTGCTTTG CTATTTACAC CACAAAGGAA AAAGCTGCAC
3601 TGCTATACAA GAAAATTATG GAAAAATATT CTGTAACCTT TATAAGTAGG CATAACAGTT
3661 ATAATCATAA CATACTGTTT TTTCTTACTC CACACAGGCA TAGAGTGTCT GCTATTAATA
3721 ACTATGCTCA AAAATTGTGT ACCTTTAGCT TTTTAATTTG TAAAGGGGTT AATAAGGAAT
3781 ATTTGATGTA TAGTGCCTTG ACTAGAGATC ATAATCAGCC ATACCACATT TGTAGAGGTT
3841 TTACTTGCTT TAAAAAACCT CCCACACCTC CCCCTGAACC TGAAACATAA AATGAATGCA
3901 ATTGTTGTTG TTAACTTGTT TATTGCAGCT TATAATGGTT ACAAATAAAG CAATAGCATC
3961 ACAAATTTCA CAAATAAAGC ATTTTTTTCA CTGCATTCTA GTTGTGGTTT GTCCAAACTC
4021 ATCAATGTAT CTTATCATGT CTGGATCGAT CCCCGGGTAC
//
```

Figure 20: Nucleic acid sequence of the known segment of the R15/appa plasmid (including the vector sequences of pBLCAT3 (SEQ ID NO:4).

```
LOCUS       R15/appa        6116 bp    DNA              SYN         15-APR-2000
DEFINITION  R15/appa transgene  with vector
ACCESSION   R15/appa
REFERENCE   1  (bases 1 to 6116)
SOURCE      synthetic construct.
      ORGANISM  synthetic construct
                artificial sequence.
KEYWORDS    salivary proline-rich protein, acid glucose-1-phosphatase; appA
            gene; periplasmic phosphoanhydride phosphohydrolase; artificial
            sequence;
AUTHORS     Golovan, S., Forsberg, C.W., Phillips, J.
  JOURNAL   Unpublished.

    DEFINITION  Rat salivary proline-rich protein (RP15) gene.
    ACCESSION   M64793 M36414
    VERSION     M64793.1  GI:206711
    SOURCE      Rat (Sprague-Dawley) liver DNA.
      ORGANISM  Rattus norvegicus
                Eukaryota; Metazoa; Chordata; Craniata; Vertebrata;
Mammalia;
                Eutheria; Rodentia; Sciurognathi; Muridae; Murinae;
Rattus.
    REFERENCE   1  (bases 1 to 1748)
      AUTHORS   Lin,H.H. and Ann,D.K.
      TITLE     Molecular characterization of rat multigene family
encoding
                proline-rich proteins
      JOURNAL   Genomics 10, 102-113 (1991)
      MEDLINE   91257817
    FEATURES             Location/Qualifiers
        source           1..1748
                         /organism="Rattus norvegicus"
                         /strain="Sprague-Dawley"
                         /db_xref="taxon:10116"
                         /tissue_type="liver"
                         /tissue_lib="cosmid genomic library"
        misc_feature             1802-1810
                         /function=" consensus sequence for initiation in
                                    higher eukaryotes "


FEATURES             Location/Qualifiers
DEFINITION  E. coli periplasmic phosphoanhydride phosphohydrolase (appA)
gene,

      ACCESSION   M58708 L03370 L03371 L03372 L03373 L03374 L03375
      VERSION     M58708.1  GI:145283
      SOURCE      Escherichia coli DNA.
      ORGANISM    Escherichia coli
            Bacteria; Proteobacteria; gamma subdivision; Enterobacteriaceae;
            Escherichia.

REFERENCE   1   (bases 1811..3109)
      AUTHORS   Dassa,J., Marck,C. and Boquet,P.L.
      TITLE     The complete nucleotide sequence of the Escherichia coli gene appA
                reveals significant homology between pH 2.5 acid phosphatase
                and glucose-1-phosphatase
      JOURNAL   J. Bacteriol. 172 (9), 5497-5500 (1990)
```

Figure 20 (continued):

```
      MEDLINE   90368616

FEATURES                  Location/Qualifiers
      Source                    1811..3109
                          /organism="Escherichia coli"
                          /db_xref="taxon:562"
      sig_peptide         1811.. 1876
      /gene="appA"
      CDS                  1811..3109
                                /gene="appA"
                          /standard_name="acid phosphatase/phytase"
                          /transl_table=11
                          /product="periplasmic phosphoanhydride phosphohydrolase"
                          /protein_id="AAA72086.1"
                          /db_xref="GI:145285"

/translation="MKAILIPFLSLLIPLTPQSAFAQSEPELKLESVVIVSRHGVRAP

TKATQLMQDVTPDAWPTWPVKLGWLTPRGGELIAYLGHYQRQRLVADGLLAKKGCPQS

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NVTDAILSRAGGSIADFTGHRQTAFRELERVLNFPQSNLCLKREKQDESCSLTQALPS

ELKVSADNVSLTGAVSLASMLTEIFLLQQAQGMPEPGWGRITDSHQWNTLLSLHNAQF

YLLQRTPEVARSRATPLLDLIKTALTPHPPQKQAYGVTLPTSVLFIAGHDTNLANLGG

ALELNWTLPGQPDNTPPGGELVFERWRRLSDNSQWIQVSLVFQTLQQMRDKTPLSLNT
                          PPGEVKLTLAGCEERNAQGMCSLAGFTQIVNEARIPACSL"
      mat_peptide         1877   3106
                          /gene="appA"
                          /product="periplasmic phosphoanhydride phosphohydrolase"

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                    /standard_name="A3 mutant"
                    /note="created by site directed mutagenesis"
                    /phenotype="silent mutation"
      mutation      replace(3092..3094," ccg changed to ccc")
                    /gene="appA"
                    /standard_name=" P428 mutant"
                    /note="created by site directed mutagenesis"
                    /phenotype=" silent mutation "
      mutation      replace(3095..3097," gcg changed to gct")
                    /gene="appA"
                    /standard_name=" A429 mutant"
                    /note="created by site directed mutagenesis"
                    /phenotype=" silent mutation "


DEFINITION  Plasmid pBLCAT3  (bases 3109 to 6116)
      ACCESSION   X64409
      VERSION     X64409.1  GI:58163
      SOURCE      synthetic construct.
        ORGANISM  synthetic construct
                  artificial sequence.
      REFERENCE   1  (bases 3109 to 6116)
        AUTHORS   Luckow,B.H.R.
        TITLE     Direct Submission
        JOURNAL   Submitted (06-FEB-1992) B.H.R. Luckow, German Cancer Res
                  Center, Im Neuenheimer Feld 280, W-6900 Heidelberg, FRG
```

Figure 20 (continued):

```
     REFERENCE   2  (bases 3109 to 6116)
       AUTHORS   Luckow,B. and Schutz,G.
       TITLE     CAT constructions with multiple unique restriction sites
for
                 the functional analysis of eukaryotic promoters and
regulatory
                 elements
       JOURNAL   Nucleic Acids Res. 15 (13), 5490 (1987)
       MEDLINE   87260024
     COMMENT     Promoterless CAT vector for transient transfection
experiments
                  with eukaryotic cells. Allows the analysis of foreign
                  promoters and  enhancers.
     FEATURES             Location/Qualifiers
          source          3109 to 6116
                          /organism="synthetic construct"
                          /db_xref="taxon:32630"
          polyA_signal    3262..3457
                          /note="SV40 signals"

          CDS             complement(4654..5514)
                          /codon_start=1
                          /transl_table=11
                          /gene="Amp"
                          /product="beta-lactamase"
                          /protein_id="CAA45753.1"
                          /db_xref="GI:58165"

BASE COUNT     1724 a   1386 c   1407 g   1599 t
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        1 GGATCCCCTT TGCTATGTAG TTTTTAATGG AAATTACAAC CCATAGTGTG TTGATAAATA
       61 GAGAGTCCTG TTTGGTTTAA GCAACCTCTG TTTCTCATAA ACTCCATAAA AACAGGAATA
      121 CTCTTTGTTT CTAGCATAAC CAAAAGATTT AGTGAATTGA AAACAATGTT CCCTTAGAGT
      181 ATAGGTCTAA TAACCCCGAA AATATTACCA TGATACTGAG CATTTGTAAG TATCTCATAG
      241 CATGTAGTAT CCATAGTCCA TCAATGAGAG AGACATTTAA CATGATTTTC ATTAATCAGG
      301 TGGAAAAGAC ATGACAACAT TCACAGGCAC TGCACAGAAC ATAGTGGTCC ACCTTGCACA
      361 TATTTCACTA AACTAGGTTT ATCTATTTTG TTGCTTTCTC TAACATCTCT GCAATGAAGC
      421 AGGTCAACAG TGCCACATAT CCTTTACTTA ACCTAAGGAA CACAAAAAAT TTTCTACATA
      481 TATCCTGGTT AGAGAGTGCT TAAAATAAGT TTTCCAAGAA TGGAAAAGAA ATGTTCTGAC
      541 TTAACAATTA AGACAGTATT TATTTAAAGC AAGAAATATG AGGCACACAA GAAAATATTT
      601 TGGGAAGAAA CCATTTGGTG AACAATATTT CAAATAAAAA TAGACAAACA TAGTTAATTG
      661 TAAAACATAT GTTTGACCAG CCCTTCTTTT CAATAGGCTT AATGTGAATA AAATGTTAAA
      721 GATTCTCTTT GGGTGGCTGC AAATTGTCCA CGAATAAGAC AAAATATAAA AATAAGGACT
      781 GAGTCTCACA AAATGAAAAG GAAATATATT CAGAAAGAGA ATCTTGAGAG AATGTGTTGT
      841 CACAAATTAA AGAAAACCTG TGGTGAATGA CATCCTGAGG CCTGAGCTAT TACTGACATT
      901 TAAGATAAAG GTAACTGTAT ACATTTGTCC CATTGAGGGG ACAAGAAAGC TGCTCTCATG
      961 TTCAGCTCTA TAATTCTTGC CTTAAACAAC TTAAATAGAA TGATTTAAAA TATGGAGCTG
     1021 TCCATGGACC TTTGAAATAT AAAATAGTCA AGCAACTTAT CAAGGAATTA CAGATTCCTT
     1081 GATACTAACA CAGGTAAATC CCACACGTGT TTTGAGACTA CATTTGCTGG GATTTTATTG
     1141 ATGTAATAGG TCACATGTTT TTCGGGCCAA TGTTGCTGTT ATTCGGTTAC TTCAAGAGAA
     1201 TAGTGGCAAC TGATGCTATG TATTCTAGGG GTTTGAAGTG ATGTTTCATG ATTGAAATTT
     1261 GTAAAAGAAT AACATCATCA TTCTTAACAA TAGAACATAT AAAGTCACAC AGAAGTGACA
     1321 GTGTTTAAGC TGTACTATTG ATCAAAGAAA TTTATTACCT TCAGTTTCAA TGGAAATAAT
     1381 TACTGATAAT ACAAACATGT GTGAACACAC ACTAATCCTA TCCAAATGCA CAGTGATACA
     1441 CAGAAAATAT TAGCAAGTAG AATGCAATAT TTATATAACG ATTGTATTTA TCAATCAATT
     1501 GTATGTATCA ATATATGGGC TATTTTCTTA CACATGATTT TATTCAAATT TACTCTAATC
     1561 ATTGTTGAAC CATTTAGAAA AGGCATACTG GCAACTTTTC CTTACCTCAT CCAGCTGGGC
     1621 AAAAGTCCCA GTGTGGAGTA AAGGATGCAA GATTTCCTGC TCTGTTAAGT ATAAAATAAT
```

Figure 20 (continued):

```
1681 AGTATGAATT CAAAGGTGCC ATTCTTCTGC TTCTAGTTAT AAAGGCAGTG CTTGCTTCTT
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1801 AGCCGCCACC ATGAAAGCCA TCTTAATCCC ATTTTTATCT CTTCTGATTC CGTTAACCCC
1861 GCAATCTGCA TTCGCTCAGA GTGAGCCGGA GCTGAAGCTG GAAAGTGTGG TGATTGTCAG
1921 TCGTCATGGT GTGCGTGCTC CAACCAAGGC CACGCAACTG ATGCAGGATG TCACCCCAGA
1981 CGCATGGCCA ACCTGGCCGG TAAAACTGGG TTGGCTGACA CCGCGCGGTG GTGAGCTAAT
2041 CGCCTATCTC GGACATTACC AACGCCAGCG TCTGGTAGCC GACGGATTGC TGGCGAAAAA
2101 GGGCTGCCCG CAGTCTGGTC AGGTCGCGAT TATTGCTGAT GTCGACGAGC GTACCCGTAA
2161 AACAGGCGAA GCCTTCGCCG CCGGGCTGGC ACCTGACTGT GCAATAACCG TACATACCCA
2221 GGCAGATACG TCCAGTCCCG ATCCGTTATT TAATCCTCTA AAAACTGGCG TTTGCCAACT
2281 GGATAACGCG AACGTGACTG ACGCGATCCT CAGCAGGGCA GGAGGGTCAA TTGCTGACTT
2341 TACCGGGCAT CGGCAAACGG CGTTTCGCGA ACTGGAACGG GTGCTTAATT TTCCGCAATC
2401 AAACTTGTGC CTTAAACGTG AGAAACAGGA CGAAAGCTGT TCATTAACGC AGGCATTACC
2461 ATCGGAACTC AAGGTGAGCG CCGACAATGT CTCATTAACC GGTGCGGTAA GCCTCGCATC
2521 AATGCTGACG GAGATATTTC TCCTGCAACA AGCACAGGGA ATGCCGGAGC CGGGGTGGGG
2581 AAGGATCACC GATTCACACC AGTGGAACAC CTTGCTAAGT TTGCATAACG CGCAATTTTA
2641 TTTGCTACAA CGCACGCCAG AGGTTGCCCG CAGCCGCGCC ACCCCGTTAT TAGATTTGAT
2701 CAAGACAGCG TTGACGCCCC ATCCACCGCA AAAACAGGCG TATGGTGTGA CATTACCCAC
2761 TTCAGTGCTG TTTATCGCCG GACACGATAC TAATCTGGCA AATCTCGGCG GCGCACTGGA
2821 GCTCAACTGG ACGCTTCCCG GTCAGCCGGA TAACACGCCG CCAGGTGGTG AACTGGTGTT
2881 TGAACGCTGG CGTCGGCTAA GCGATAACAG CCAGTGGATT CAGGTTTCGC TGGTCTTCCA
2941 GACTTTACAG CAGATGCGTG ATAAAACGCC GCTGTCATTA AATACGCCGC CCGGAGAGGT
3001 GAAACTGACC CTGGCAGGAT GTGAAGAGCG AAATGCGCAG GGCATGTGTT CGTTGGCAGG
3061 TTTTACGCAA ATCGTGAATG AAGCACGCAT ACCCGCTTGC AGTTTGTAAG GTATAAGGCA
3121 GTTATTGGTG CCCTTAAACG CCTGGTGCTA CGCCTGAATA AGTGATAATA AGCGGATGAA
3181 TGGCAGAAAT TCGCCGGATC TTTGTGAAGG AACCTTACTT CTGTGGTGTG ACATAATTGG
3241 ACAAACTACC TACAGAGATT TAAAAAACCT CCCACACCTC CCCCTGAACC TGAAACATAA
3301 AATGAATGCA ATTGTTGTTG TTAACTTGTT TATTGCAGCT TATAATGGTT ACAAATAAAG
3361 CAATAGCATC ACAAATTTCA CAAATAAAGC ATTTTTTTCA CTGCATTCTA GTTGTGGTTT
3421 GTCCAAACTC ATCAATGTAT CTTATCATGT CTGGATCGAT CCCCGGGTAC CGAGCTCGAA
3481 TTCGTAATCA TGGTCATAGC TGTTTCCTGT GTGAAATTGT TATCCGCTCA CAATTCCACA
3541 CAACATACGA GCCGGAAGCA TAAAGTGTAA AGCCTGGGGT GCCTAATGAG TGAGCTAACT
3601 CACATTAATT GCGTTGCGCT CACTGCCCGC TTTCCAGTCG GGAAACCTGT CGTGCCAGCT
3661 GCATTAATGA ATCGGCCAAC GCGCGGGGAG AGGCGGTTTG CGTATTGGGC GCTCTTCCGC
3721 TTCCTCGCTC ACTGACTCGC TGCGCTCGGT CGTTCGGCTG CGGCGAGCGG TATCAGCTCA
3781 CTCAAAGGCG GTAATACGGT TATCCACAGA ATCAGGGGAT AACGCAGGAA AGAACATGTG
3841 AGCAAAAGGC CAGCAAAAGG CCAGGAACCG TAAAAAGGCC GCGTTGCTGG CGTTTTTCCA
3901 TAGGCTCCGC CCCCCTGACG AGCATCACAA AAATCGACGC TCAAGTCAGA GGTGGCGAAA
3961 CCCGACAGGA CTATAAAGAT ACCAGGCGTT TCCCCCTGGA AGCTCCCTCG TGCGCTCTCC
4021 TGTTCCGACC CTGCCGCTTA CCGGATACCT GTCCGCCTTT CTCCCTTCGG GAAGCGTGGC
4081 GCTTTCTCAA TGCTCACGCT GTAGGTATCT CAGTTCGGTG TAGGTCGTTC GCTCCAAGCT
4141 GGGCTGTGTG CACGAACCCC CCGTTCAGCC CGACCGCTGC GCCTTATCCG GTAACTATCG
4201 TCTTGAGTCC AACCCGGTAA GACACGACTT ATCGCCACTG GCAGCAGCCA CTGGTAACAG
4261 GATTAGCAGA GCGAGGTATG TAGGCGGTGC TACAGAGTTC TTGAAGTGGT GGCCTAACTA
4321 CGGCTACACT AGAAGGACAG TATTTGGTAT CTGCGCTCTG CTGAAGCCAG TTACCTTCGG
4381 AAAAAGAGTT GGTAGCTCTT GATCCGGCAA ACAAACCACC GCTGGTAGCG GTGGTTTTTT
4441 TGTTTGCAAG CAGCAGATTA CGCGCAGAAA AAAAGGATCT CAAGAAGATC CTTTGATCTT
4501 TTCTACGGGG TCTGACGCTC AGTGGAACGA AAACTCACGT TAAGGGATTT TGGTCATGAG
4561 ATTATCAAAA AGGATCTTCA CCTAGATCCT TTTAAATTAA AAATGAAGTT TTAAATCAAT
4621 CTAAAGTATA TATGAGTAAA CTTGGTCTGA CAGTTACCAA TGCTTAATCA GTGAGGCACC
4681 TATCTCAGCG ATCTGTCTAT TTCGTTCATC CATAGTTGCC TGACTCCCCG TCGTGTAGAT
4741 AACTACGATA CGGGAGGGCT TACCATCTGG CCCCAGTGCT GCAATGATAC CGCGAGACCC
4801 ACGCTCACCG GCTCCAGATT TATCAGCAAT AAACCAGCCA GCCGGAAGGG CCGAGCGCAG
4861 AAGTGGTCCT GCAACTTTAT CCGCCTCCAT CCAGTCTATT AATTGTTGCC GGGAAGCTAG
4921 AGTAAGTAGT TCGCCAGTTA ATAGTTTGCG CAACGTTGTT GCCATTGCTA CAGGCATCGT
4981 GGTGTCACGC TCGTCGTTTG GTATGGCTTC ATTCAGCTCC GGTTCCCAAC GATCAAGGCG
5041 AGTTACATGA TCCCCCATGT TGTGCAAAAA AGCGGTTAGC TCCTTCGGTC CTCCGATCGT
5101 TGTCAGAAGT AAGTTGGCCG CAGTGTTATC ACTCATGGTT ATGGCAGCAC TGCATAATTC
```

Figure 20 (continued):

```
5161 TCTTACTGTC ATGCCATCCG TAAGATGCTT TTCTGTGACT GGTGAGTACT CAACCAAGTC
5221 ATTCTGAGAA TAGTGTATGC GGCGACCGAG TTGCTCTTGC CCGGCGTCAA TACGGGATAA
5281 TACCGCGCCA CATAGCAGAA CTTTAAAAGT GCTCATCATT GGAAAACGTT CTTCGGGGCG
5341 AAAACTCTCA AGGATCTTAC CGCTGTTGAG ATCCAGTTCG ATGTAACCCA CTCGTGCACC
5401 CAACTGATCT TCAGCATCTT TTACTTTCAC CAGCGTTTCT GGGTGAGCAA AAACAGGAAG
5461 GCAAAATGCC GCAAAAAAGG GAATAAGGGC GACACGGAAA TGTTGAATAC TCATACTCTT
5521 CCTTTTTCAA TATTATTGAA GCATTTATCA GGGTTATTGT CTCATGAGCG GATACATATT
5581 TGAATGTATT TAGAAAAATA AACAAATAGG GGTTCCGCGC ACATTTCCCC GAAAAGTGCC
5641 ACCTGACGTC TAAGAAACCA TTATTATCAT GACATTAACC TATAAAAATA GGCGTATCAC
5701 GAGGCCCTTT CGTCTCGCGC GTTTCGGTGA TGACGGTGAA AACCTCTGAC ACATGCAGCT
5761 CCCGGAGACG GTCACAGCTT GTCTGTAAGC GGATGCCGGG AGCAGACAAG CCCGTCAGGG
5821 CGCGTCAGCG GGTGTTGGCG GGTGTCGGGG CTGGCTTAAC TATGCGGCAT CAGAGCAGAT
5881 TGTACTGAGA GTGCACCATA TGCGGTGTGA AATACCGCAC AGATGCGTAA GGAGAAAATA
5941 CCGCATCAGG CGCCATTCGC CATTCAGGCT GCGCAACTGT TGGGAAGGGC GATCGGTGCG
6001 GGCCTCTTCG CTATTACGCC AGCTGGCGAA AGGGGGATGT GCTGCAAGGC GATTAAGTTG
6061 GGTAACGCCA GGGTTTTCCC AGTCACGACG TTGTAAAACG ACGGCCAGTG CCAAGC
//
```

Figure 21: Nucleic acid sequence of the known segment of the R15/appa transgene used for the generation of transgenic mice (SEQ ID NO:5).

```
LOCUS       R15/appa    3470 bp    DNA             SYN        15-APR-2000
DEFINITION  R15/appa transgene with vector sequences removed.
ACCESSION   R15/appa
REFERENCE   1  (bases 1 to 3470)
SOURCE      synthetic construct.
  ORGANISM  synthetic construct
            artificial sequence.
KEYWORDS    salivary proline-rich protein, acid glucose-1-phosphatase; appA
            gene; periplasmic phosphoanhydride phosphohydrolase; artificial
            sequence;
AUTHORS     Golovan, S., Forsberg, C.W., Phillips, J.
  JOURNAL   Unpublished.

    DEFINITION  Rat salivary proline-rich protein (RP15) gene.
    ACCESSION   M64793 M36414
    VERSION     M64793.1  GI:206711
    SOURCE      Rat (Sprague-Dawley) liver DNA.
      ORGANISM  Rattus norvegicus
                Eukaryota; Metazoa; Chordata; Craniata; Vertebrata;
Mammalia;
                Eutheria; Rodentia; Sciurognathi; Muridae; Murinae;
Rattus.
    REFERENCE   1  (bases 1 to 1748)
      AUTHORS   Lin,H.H. and Ann,D.K.
      TITLE     Molecular characterization of rat multigene family
encoding
                proline-rich proteins
      JOURNAL   Genomics 10, 102-113 (1991)
      MEDLINE   91257817
    FEATURES             Location/Qualifiers
        source           1..1748
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                         /strain="Sprague-Dawley"
                         /db_xref="taxon:10116"
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                         /tissue_lib="cosmid genomic library"
        misc_feature           1802-1810
                         /function=" consensus sequence for initiation in
                                   higher eukaryotes "


FEATURES             Location/Qualifiers

DEFINITION  E. coli periplasmic phosphoanhydride phosphohydrolase (appA)
gene,

      ACCESSION   M58708 L03370 L03371 L03372 L03373 L03374 L03375
      VERSION     M58708.1  GI:145283
      SOURCE      Escherichia coli DNA.
      ORGANISM    Escherichia coli
            Bacteria; Proteobacteria; gamma subdivision; Enterobacteriaceae;
            Escherichia.

REFERENCE   1   (bases 1811..3109)
      AUTHORS   Dassa,J., Marck,C. and Boquet,P.L.
      TITLE     The complete nucleotide sequence of the Escherichia coli gene appA
                reveals significant homology between pH 2.5 acid phosphatase
                and glucose-1-phosphatase
```

Figure 21 (continued):

```
      JOURNAL   J. Bacteriol. 172 (9), 5497-5500 (1990)
      MEDLINE   90368616

FEATURES                Location/Qualifiers
      Source                  1811..3109
                              /organism="Escherichia coli"
                              /db_xref="taxon:562"
      sig_peptide             1811..1876
                              /gene="appA"
      CDS                     1811..3109
                              /gene="appA"
                              /standard_name="acid phosphatase/phytase"
                              /transl_table=11
                              /product="periplasmic phosphoanhydride phosphohydrolase"
                              /protein_id="AAA72086.1"
                              /db_xref="GI:145285"

/translation="MKAILIPFLSLLIPLTPQSAFAQSEPELKLESVVIVSRHGVRAP

TKATQLMQDVTPDAWPTWPVKLGWLTPRGGELIAYLGHYQRQRLVADGLLAKKGCPQS

GQVAIIADVDERTRKTGEAFAAGLAPDCAITVHTQADTSSPDPLFNPLKTGVCQLDNA

NVTDAILSRAGGSIADFTGHRQTAFRELERVLNFPQSNLCLKREKQDESCSLTQALPS

ELKVSADNVSLTGAVSLASMLTEIFLLQQAQGMPEPGWGRITDSHQWNTLLSLHNAQF

YLLQRTPEVARSRATPLLDLIKTALTPHPPQKQAYGVTLPTSVLFIAGHDTNLANLGG

ALELNWTLPGQPDNTPPGGELVFERWRRLSDNSQWIQVSLVFQTLQQMRDKTPLSLNT
                        PPGEVKLTLAGCEERNAQGMCSLAGFTQIVNEARIPACSL"
      mat_peptide       1877  3106
                        /gene="appA"
                        /product="periplasmic phosphoanhydride phosphohydrolase"

      mutation          replace(1817.. 1819,"gcg changed to gcc")
                        /gene="appA"
                        /standard_name="A3 mutant"
                        /note="created by site directed mutagenesis"
                        /phenotype="silent mutation"
      mutation          replace(3092..3094," ccg changed to ccc")
                        /gene="appA"
                        /standard_name=" P428 mutant"
                        /note="created by site directed mutagenesis"
                        /phenotype=" silent mutation "
      mutation          replace(3095..3097," gcg changed to gct")
                        /gene="appA"
                        /standard_name=" A429 mutant"
                        /note="created by site directed mutagenesis"
                        /phenotype=" silent mutation "


          polyA_signal      3262..3457
                            /note="SV40 signals"

BASE COUNT      1065 a    721 c    735 g    949 t
ORIGIN
        1 GGATCCCCTT TGCTATGTAG TTTTTAATGG AAATTACAAC CCATAGTGTG TTGATAAATA
       61 GAGAGTCCTG TTTGGTTTAA GCAACCTCTG TTTCTCATAA ACTCCATAAA AACAGGAATA
      121 CTCTTTGTTT CTAGCATAAC CAAAAGATTT AGTGAATTGA AAACAATGTT CCCTTAGAGT
      181 ATAGGTCTAA TAACCCCGAA AATATTACCA TGATACTGAG CATTTGTAAG TATCTCATAG
      241 CATGTAGTAT CCATAGTCCA TCAATGAGAG AGACATTTAA CATGATTTTC ATTAATCAGG
```

Figure 21 (continued):

```
 301 TGGAAAAGAC ATGACAACAT TCACAGGCAC TGCACAGAAC ATAGTGGTCC ACCTTGCACA
 361 TATTTCACTA AACTAGGTTT ATCTATTTTG TTGCTTTCTC TAACATCTCT GCAATGAAGC
 421 AGGTCAACAG TGCCACATAT CCTTTACTTA ACCTAAGGAA CACAAAAAAT TTTCTACATA
 481 TATCCTGGTT AGAGAGTGCT TAAAATAAGT TTTCCAAGAA TGGAAAAGAA ATGTTCTGAC
 541 TTAACAATTA AGACAGTATT TATTTAAAGC AAGAAATATG AGGCACACAA GAAAATATTT
 601 TGGGAAGAAA CCATTTGGTG AACAATATTT CAAATAAAAA TAGACAAACA TAGTTAATTG
 661 TAAAACATAT GTTTGACCAG CCCTTCTTTT CAATAGGCTT AATGTGAATA AAATGTTAAA
 721 GATTCTCTTT GGGTGGCTGC AAATTGTCCA CGAATAAGAC AAAATATAAA AATAAGGACT
 781 GAGTCTCACA AAATGAAAAG GAAATATATT CAGAAAGAGA ATCTTGAGAG AATGTGTTGT
 841 CACAAATTAA AGAAAACCTG TGGTGAATGA CATCCTGAGG CCTGAGCTAT TACTGACATT
 901 TAAGATAAAG GTAACTGTAT ACATTTGTCC CATTGAGGGG ACAAGAAAGC TGCTCTCATG
 961 TTCAGCTCTA TAATTCTTGC CTTAAACAAC TTAAATAGAA TGATTTAAAA TATGGAGCTG
1021 TCCATGGACC TTTGAAATAT AAAATAGTCA AGCAACTTAT CAAGGAATTA CAGATTCCTT
1081 GATACTAACA CAGGTAAATC CCACACGTGT TTTGAGACTA CATTTGCTGG GATTTTATTG
1141 ATGTAATAGG TCACATGTTT TTCGGGCCAA TGTTGCTGTT ATTCGGTTAC TTCAAGAGAA
1201 TAGTGGCAAC TGATGCTATG TATTCTAGGG GTTTGAAGTG ATGTTTCATG ATTGAAATTT
1261 GTAAAAGAAT AACATCATCA TTCTTAACAA TAGAACATAT AAAGTCACAC AGAAGTGACA
1321 GTGTTTAAGC TGTACTATTG ATCAAAGAAA TTTATTACCT TCAGTTTCAA TGGAAATAAT
1381 TACTGATAAT ACAAACATGT GTGAACACAC ACTAATCCTA TCCAAATGCA CAGTGATACA
1441 CAGAAAATAT TAGCAAGTAG AATGCAATAT TTATATAACG ATTGTATTTA TCAATCAATT
1501 GTATGTATCA ATATATGGGC TATTTTCTTA CACATGATTT TATTCAAATT TACTCTAATC
1561 ATTGTTGAAC CATTTAGAAA AGGCATACTG GCAACTTTTC CTTACCTCAT CCAGCTGGGC
1621 AAAAGTCCCA GTGTGGAGTA AAGGATGCAA GATTTCCTGC TCTGTTAAGT ATAAAATAAT
1681 AGTATGAATT CAAAGGTGCC ATTCTTCTGC TTCTAGTTAT AAAGGCAGTG CTTGCTTCTT
1741 CCAGCACAGA TCTGGATCTC GAGGAGCTTG GCGAGATTTT CAGGAGCTAA GGAAGCTAAA
1801 AGCCGCCACC ATGAAAGCCA TCTTAATCCC ATTTTTATCT CTTCTGATTC CGTTAACCCC
1861 GCAATCTGCA TTCGCTCAGA GTGAGCCGGA GCTGAAGCTG GAAAGTGTGG TGATTGTCAG
1921 TCGTCATGGT GTGCGTGCTC CAACCAAGGC CACGCAACTG ATGCAGGATG TCACCCCAGA
1981 CGCATGGCCA ACCTGGCCGG TAAAACTGGG TTGGCTGACA CCGCGCGGTG GTGAGCTAAT
2041 CGCCTATCTC GGACATTACC AACGCCAGCG TCTGGTAGCC GACGGATTGC TGGCGAAAAA
2101 GGGCTGCCCG CAGTCTGGTC AGGTCGCGAT TATTGCTGAT GTCGACGAGC GTACCCGTAA
2161 AACAGGCGAA GCCTTCGCCG CCGGGCTGGC ACCTGACTGT GCAATAACCG TACATACCCA
2221 GGCAGATACG TCCAGTCCCG ATCCGTTATT TAATCCTCTA AAAACTGGCG TTTGCCAACT
2281 GGATAACGCG AACGTGACTG ACGCGATCCT CAGCAGGGCA GGAGGGTCAA TTGCTGACTT
2341 TACCGGGCAT CGGCAAACGG CGTTTCGCGA ACTGGAACGG GTGCTTAATT TTCCGCAATC
2401 AAACTTGTGC CTTAAACGTG AGAAACAGGA CGAAAGCTGT TCATTAACGC AGGCATTACC
2461 ATCGGAACTC AAGGTGAGCG CCGACAATGT CTCATTAACC GGTGCGGTAA GCCTCGCATC
2521 AATGCTGACG GAGATATTTC TCCTGCAACA AGCACAGGGA ATGCCGGAGC CGGGGTGGGG
2581 AAGGATCACC GATTCACACC AGTGGAACAC CTTGCTAAGT TTGCATAACG CGCAATTTTA
2641 TTTGCTACAA CGCACGCCAG AGGTTGCCCG CAGCCGCGCC ACCCCGTTAT TAGATTTGAT
2701 CAAGACAGCG TTGACGCCCC ATCCACCGCA AAAACAGGCG TATGGTGTGA CATTACCCAC
2761 TTCAGTGCTG TTTATCGCCG GACACGATAC TAATCTGGCA AATCTCGGCG GCGCACTGGA
2821 GCTCAACTGG ACGCTTCCCG GTCAGCCGGA TAACACGCCG CCAGGTGGTG AACTGGTGTT
2881 TGAACGCTGG CGTCGGCTAA GCGATAACAG CCAGTGGATT CAGGTTTCGC TGGTCTTCCA
2941 GACTTTACAG CAGATGCGTG ATAAAACGCC GCTGTCATTA AATACGCCGC CCGGAGAGGT
3001 GAAACTGACC CTGGCAGGAT GTGAAGAGCG AAATGCGCAG GGCATGTGTT CGTTGGCAGG
3061 TTTTACGCAA ATCGTGAATG AAGCACGCAT ACCCGCTTGC AGTTTGTAAG GTATAAGGCA
3121 GTTATTGGTG CCCTTAAACG CCTGGTGCTA CGCCTGAATA AGTGATAATA AGCGGATGAA
3181 TGGCAGAAAT TCGCCGGATC TTTGTGAAGG AACCTTACTT CTGTGGTGTG ACATAATTGG
3241 ACAAACTACC TACAGAGATT TAAAAAACCT CCCACACCTC CCCCTGAACC TGAAACATAA
3301 AATGAATGCA ATTGTTGTTG TTAACTTGTT TATTGCAGCT TATAATGGTT ACAAATAAAG
3361 CAATAGCATC ACAAATTTCA CAAATAAAGC ATTTTTTTCA CTGCATTCTA GTTGTGGTTT
3421 GTCCAAACTC ATCAATGTAT CTTATCATGT CTGGATCGAT CCCCGGGTAC
```

//

Figure 22: Nucleic acid sequence of the SV40/APPA+intron plasmid (SEQ ID NO:6).

```
LOCUS       SV40/APPA    5421 bp    DNA   CIRCULAR SYN        14-APR-2000
DEFINITION  Ligation of SV40 promoter/enhancer into CAT/APPA+intron
ACCESSION   SV40/APPA
REFERENCE   1  (bases 1 to 5421)
SOURCE      synthetic construct.
      ORGANISM  synthetic construct
                artificial sequence.
KEYWORDS    SV40 promoter/enhancer, acid glucose-1-phosphatase; appA gene;
            periplasmic phosphoanhydride phosphohydrolase; artificial
            sequence;
AUTHORS     Golovan, S., Forsberg, C.W., Phillips, J.
JOURNAL  Unpublished.

DEFINITION  E. coli periplasmic phosphoanhydride phosphohydrolase (appA)
gene,

      ACCESSION    M58708 L03370 L03371 L03372 L03373 L03374 L03375
      VERSION      M58708.1  GI:145283
      SOURCE       Escherichia coli DNA.
      ORGANISM     Escherichia coli
           Bacteria; Proteobacteria; gamma subdivision; Enterobacteriaceae;
           Escherichia.

REFERENCE   1   (bases 40 1337)
      AUTHORS   Dassa,J., Marck,C. and Boquet,P.L.
      TITLE     The complete nucleotide sequence of the Escherichia coli gene appA
                reveals significant homology between pH 2.5 acid phosphatase
                and glucose-1-phosphatase
      JOURNAL   J. Bacteriol. 172 (9), 5497-5500 (1990)
      MEDLINE   90368616

FEATURES                  Location/Qualifiers
      Source              40  1337
                          /organism="Escherichia coli"
                          /db_xref="taxon:562"
      sig_peptide         40..105
                          /gene="appA"
      CDS                  40  1337
                               /gene="appA"
      /standard_name="acid phosphatase/phytase"
                          /transl_table=11
                          /product="periplasmic phosphoanhydride phosphohydrolase"
                          /protein_id="AAA72086.1"
                          /db_xref="GI:145285"

/translation="MKAILIPFLSLLIPLTPQSAFAQSEPELKLESVVIVSRHGVRAP

TKATQLMQDVTPDAWPTWPVKLGWLTPRGGELIAYLGHYQRQRLVADGLLAKKGCPQS

GQVAIIADVDERTRKTGEAFAAGLAPDCAITVHTQADTSSPDPLFNPLKTGVCQLDNA

NVTDAILSRAGGSIADFTGHRQTAFRELERVLNFPQSNLCLKREKQDESCSLTQALPS

ELKVSADNVSLTGAVSLASMLTEIFLLQQAQGMPEPGWGRITDSHQWNTLLSLHNAQF

YLLQRTPEVARSRATPLLDLIKTALTPHPPQKQAYGVTLPTSVLFIAGHDTNLANLGG

ALELNWTLPGQPDNTPPGGELVFERWRRLSDNSQWIQVSLVFQTLQQMRDKTPLSLNT
                          PPGEVKLTLAGCEERNAQGMCSLAGFTQIVNEARIPACSL"
       mat_peptide        106    1334
                          /gene="appA"
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Figure 22 (continued):

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                        /product="periplasmic phosphoanhydride phosphohydrolase"

      mutation      replace(46.. 48,"gcg changed to gcc")
                    /gene="appA"
                    /standard_name="A3 mutant"
                    /note="created by site directed mutagenesis"
                    /phenotype="silent mutation"
      mutation      replace(1320..1322," ccg changed to ccc")
                    /gene="appA"
                    /standard_name=" P428 mutant"
                    /note="created by site directed mutagenesis"
                    /phenotype=" silent mutation "
      mutation      replace(1323..1325," gcg changed to gct")
                    /gene="appA"
                    /standard_name=" A429 mutant"
                    /note="created by site directed mutagenesis"
                    /phenotype=" silent mutation "


DEFINITION  Plasmid pBLCAT3  (bases 2200 to 4924)
     ACCESSION   X64409
     VERSION     X64409.1  GI:58163
     SOURCE      synthetic construct.
       ORGANISM  synthetic construct
                 artificial sequence.
     REFERENCE   1  (bases 2200 to 4924)
       AUTHORS   Luckow,B.H.R.
       TITLE     Direct Submission
       JOURNAL   Submitted (06-FEB-1992) B.H.R. Luckow, German Cancer Res
                 Center, Im Neuenheimer Feld 280, W-6900 Heidelberg, FRG
     REFERENCE   2  (bases 2200 to 4924)
       AUTHORS   Luckow,B. and Schutz,G.
       TITLE     CAT constructions with multiple unique restriction sites
for
                 the functional analysis of eukaryotic promoters and
regulatory
                 elements
       JOURNAL   Nucleic Acids Res. 15 (13), 5490 (1987)
       MEDLINE   87260024
     COMMENT     Promoterless CAT vector for transient transfection
experiments
                  with eukaryotic cells. Allows the analysis of foreign
                  promoters and  enhancers.
     FEATURES             Location/Qualifiers
          source          2200 to 4924
                          /organism="synthetic construct"
                          /db_xref="taxon:32630"

          SV40 t intron   1380..1993
                          /note="SV40 signals"
          polyA_signal    1990..2230
                          /note="SV40 signals"
          CDS             complement(3471..4317)
                          /codon_start=1
                          /transl_table=11
                          /gene="Amp"
                          /product="beta-lactamase"
                          /protein_id="CAA45753.1"
                          /db_xref="GI:58165"
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Figure 22 (continued):

```
     SV40 promoter/enhancer   5023..5402
                      /note="SV40 signals"


BASE COUNT      1413 a    1321 c    1331 g    1355 t
ORIGIN
        1 CGAGATTTTC AGGAGCTAAG GAAGCTAAAA GCCGCCACCA TGAAAGCCAT CTTAATCCCA
       61 TTTTTATCTC TTCTGATTCC GTTAACCCCG CAATCTGCAT TCGCTCAGAG TGAGCCGGAG
      121 CTGAAGCTGG AAAGTGTGGT GATTGTCAGT CGTCATGGTG TGCGTGCTCC AACCAAGGCC
      181 ACGCAACTGA TGCAGGATGT CACCCCAGAC GCATGGCCAA CCTGGCCGGT AAAACTGGGT
      241 TGGCTGACAC CGCGNGGTGG TGAGCTAATC GCCTATCTCG GACATTACCA ACGCCAGCGT
      301 CTGGTAGCCG ACGGATTGCT GGCGAAAAAG GGCTGCCCGC AGTCTGGTCA GGTCGCGATT
      361 ATTGCTGATG TCGACGAGCG TACCCGTAAA ACAGGCGAAG CCTTCGCCGC CGGGCTGGCA
      421 CCTGACTGTG CAATAACCGT ACATACCCAG GCAGATACGT CCAGTCCCGA TCCGTTATTT
      481 AATCCTCTAA AAACTGGCGT TTGCCAACTG GATAACGCGA ACGTGACTGA CGCGATCCTC
      541 AGCAGGGCAG GAGGGTCAAT TGCTGACTTT ACCGGGCATC GGCAAACGGC GTTTCGCGAA
      601 CTGGAACGGG TGCTTAATTT TCCGCAATCA AACTTGTGCC TTAAACGTGA GAAACAGGAC
      661 GAAAGCTGTT CATTAACGCA GGCATTACCA TCGGAACTCA AGGTGAGCGC CGACAATGTC
      721 TCATTAACCG GTGCGGTAAG CCTCGCATCA ATGCTGACGG AGATATTTCT CCTGCAACAA
      781 GCACAGGGAA TGCCGGAGCC GGGGTGGGGA AGGATCACCG ATTCACACCA GTGGAACACC
      841 TTGCTAAGTT TGCATAACGC GCAATTTTAT TTGCTACAAC GCACGCCAGA GGTTGCCCGC
      901 AGCCGCGCCA CCCCGTTATT AGATTTGATC AAGACAGCGT TGACGCCCCA CCACCGCAAA
      961 AACAGGCGTA TGGTGTGACA TTACCCACTT CAGTGCTGTT TATCGCCGGA CACGATACTA
     1021 ATCTGGCAAA TCTCGGCGGC GCACTGGAGC TCAACTGGAC GCTTCCCGGT CAGCCGGATA
     1081 ACACGCCGCC AGGTGGTGAA CTGGTGTTTG AACGCTGGCG TCGGCTAAGC GATAACAGCC
     1141 AGTGGATTCA GGTTTCGCTG GTCTTCCAGA CTTTACAGCA GATGCGTGAT AAAACGCCGC
     1201 TGTCATTAAA TACGCCGCCC GGAGAGGTGA AACTGACCCT GGCAGGATGT GAAGAGCGAA
     1261 ATGCGCAGGG CATGTGTTCG TTGGCAGGTT TTACGCAAAT CGTGAATGAA GCACGCATAC
     1321 CCGCTTGCAG TTTGTAAGGC AGTTATTGGT GCCCTTAAAC GCCTGGTGCT ACGCCTGAAT
     1381 AAGTGATAAT AAGCGGATGA ATGGCAGAAA TTCGCCGGAT CTTTGTGAAG GAACCTTACT
     1441 TCTGTGGTGT GACATAATTG GACAAACTAC CTACAGAGAT TTAAAGCTCT AAGGTAAATA
     1501 TAAAATTTTT AAGTGTATAA TGTGTTAAAC TACTGATTCT AATTGTTTGT GTATTTTAGA
     1561 TTCCAACCTA TGGAACTGAT GAATGGGAGC AGTGGTGGAA TGCCTTTAAT GAGGAAAACC
     1621 TGTTTTGCTC AGAAGAAATG CCATCTAGTG ATGATGAGGC TACTGCTGAC TCTCAACATT
     1681 CTACTCCTCC AAAAAAGAAG AGAAAGGTAG AAGACCCCAA GGACTTTCCT TCAGAATTGC
     1741 TAAGTTTTTT GAGTCATGCT GTGTTTAGTA ATAGAACTCT TGCTTGCTTT GCTATTTACA
     1801 CCACAAAGGA AAAAGCTGCA CTGCTATACA AGAAAATTAT GGAAAAATAT TCTGTAACCT
     1861 TTATAAGTAG GCATAACAGT TATAATCATA ACATACTGTT TTTTCTTACT CCACACAGGC
     1921 ATAGAGTGTC TGCTATTAAT AACTATGCTC AAAAATTGTG TACCTTTAGC TTTTTAATTT
     1981 GTAAAGGGGT TAATAAGGAA TATTTGATGT ATAGTGCCTT GACTAGAGAT CATAATCAGC
     2041 CATACCACAT TTGTAGAGGT TTTACTTGCT TTAAAAAACC TCCCACACCT CCCCCTGAAC
     2101 CTGAAACATA AAATGAATGC AATTGTTGTT GTTAACTTGT TTATTGCAGC TTATAATGGT
     2161 TACAAATAAA GCAATAGCAT CACAAATTTC ACAAATAAAG CATTTTTTTC ACTGCATTCT
     2221 AGTTGTGGTT TGTCCAAACT CATCAATGTA TCTTATCATG TCTGGATCGA TCCCCGGGTA
     2281 CCGAGCTCGA ATTCGTAATC ATGGTCATAG CTGTTTCCTG TGTGAAATTG TTATCCGCTC
     2341 ACAATTCCAC ACAACATACG AGCCGGAAGC ATAAAGTGTA AAGCCTGGGG TGCCTAATGA
     2401 GTGAGCTAAC TCACATTAAT TGCGTTGCGC TCACTGCCCG CTTTCCAGTC GGGAAACCTG
     2461 TCGTGCCAGC TGCATTAATG AATCGGCCAA CGCGCGGGGA GAGGCGGTTT GCGTATTGGG
     2521 CGCTCTTCCG CTTCCTCGCT CACTGACTCG CTGCGCTCGG TCGTTCGGCT GCGGCGAGCG
     2581 GTATCAGCTC ACTCAAAGGC GGTAATACGG TTATCCACAG AATCAGGGGA TAACGCAGGA
     2641 AAGAACATGT GAGCAAAAGG CCAGCAAAAG GCCAGGAACC GTAAAAAGGC CGCGTTGCTG
     2701 GCGTTTTTCC ATAGGCTCCG CCCCCCTGAC GAGCATCACA AAAATCGACG CTCAAGTCAG
     2761 AGGTGGCGAA ACCCGACAGG ACTATAAAGA TACCAGGCGT TTCCCCCTGG AAGCTCCCTC
     2821 GTGCGCTCTC CTGTTCCGAC CCTGCCGCTT ACCGGATACC TGTCCGCCTT TCTCCCTTCG
     2881 GGAAGCGTGG CGCTTTCTCA ATGCTCACGC TGTAGGTATC TCAGTTCGGT GTAGGTCGTT
     2941 CGCTCCAAGC TGGGCTGTGT GCACGAACCC CCCGTTCAGC CCGACCGCTG CGCCTTATCC
     3001 GGTAACTATC GTCTTGAGTC CAACCCGGTA AGACACGACT TATCGCCACT GGCAGCAGCC
     3061 ACTGGTAACA GGATTAGCAG AGCGAGGTAT GTAGGCGGTG CTACAGAGTT CTTGAAGTGG
```

Figure 22 (continued):

```
3121 TGGCCTAACT ACGGCTACAC TAGAAGGACA GTATTTGGTA TCTGCGCTCT GCTGAAGCCA
3181 GTTACCTTCG GAAAAAGAGT TGGTAGCTCT TGATCCGGCA AACAAACCAC CGCTGGTAGC
3241 GGTGGTTTTT TTGTTTGCAA GCAGCAGATT ACGCGCAGAA AAAAAGGATC TCAAGAAGAT
3301 CCTTTGATCT TTTCTACGGG GTCTGACGCT CAGTGGAACG AAAACTCACG TTAAGGGATT
3361 TTGGTCATGA GATTATCAAA AAGGATCTTC ACCTAGATCC TTTTAAATTA AAAATGAAGT
3421 TTTAAATCAA TCTAAAGTAT ATATGAGTAA ACTTGGTCTG ACAGTTACCA ATGCTTAATC
3481 AGTGAGGCAC CTATCTCAGC GATCTGTCTA TTTCGTTCAT CCATAGTTGC CTGACTCCCC
3541 GTCGTGTAGA TAACTACGAT ACGGGAGGGC TTACCATCTG GCCCCAGTGC TGCAATGATA
3601 CCGCGAGACC CACGCTCACC GGCTCCAGAT TTATCAGCAA TAAACCAGCC AGCCGGAAGG
3661 GCCGAGCGCA GAAGTGGTCC TGCAACTTTA TCCGCCTCCA TCCAGTCTAT TAATTGTTGC
3721 CGGGAAGCTA GAGTAAGTAG TTCGCCAGTT AATAGTTTGC GCAACGTTGT TGCCATTGCT
3781 ACAGGCATCG TGGTGTCACG CTCGTCGTTT GGTATGGCTT CATTCAGCTC CGGTTCCCAA
3841 CGATCAAGGC GAGTTACATG ATCCCCCATG TTGTGCAAAA AAGCGGTTAG CTCCTTCGGT
3901 CCTCCGATCG TTGTCAGAAG TAAGTTGGCC GCAGTGTTAT CACTCATGGT TATGGCAGCA
3961 CTGCATAATT CTCTTACTGT CATGCCATCC GTAAGATGCT TTTCTGTGAC TGGTGAGTAC
4021 TCAACCAAGT CATTCTGAGA ATAGTGTATG CGGCGACCGA GTTGCTCTTG CCCGGCGTCA
4081 ATACGGGATA ATACCGCGCC ACATAGCAGA ACTTTAAAAG TGCTCATCAT TGGAAAACGT
4141 TCTTCGGGGC GAAAACTCTC AAGGATCTTA CCGCTGTTGA GATCCAGTTC GATGTAACCC
4201 ACTCGTGCAC CCAACTGATC TTCAGCATCT TTTACTTTCA CCAGCGTTTC TGGGTGAGCA
4261 AAAACAGGAA GGCAAAATGC CGCAAAAAAG GGAATAAGGG CGACACGGAA ATGTTGAATA
4321 CTCATACTCT TCCTTTTTCA ATATTATTGA AGCATTTATC AGGGTTATTG TCTCATGAGC
4381 GGATACATAT TTGAATGTAT TTAGAAAAAT AAACAAATAG GGGTTCCGCG CACATTTCCC
4441 CGAAAAGTGC CACCTGACGT CTAAGAAACC ATTATTATCA TGACATTAAC CTATAAAAAT
4501 AGGCGTATCA CGAGGCCCTT TCGTCTCGCG CGTTTCGGTG ATGACGGTGA AAACCTCTGA
4561 CACATGCAGC TCCCGGAGAC GGTCACAGCT TGTCTGTAAG CGGATGCCGG GAGCAGACAA
4621 GCCCGTCAGG GCGCGTCAGC GGGTGTTGGC GGGTGTCGGG GCTGGCTTAA CTATGCGGCA
4681 TCAGAGCAGA TTGTACTGAG AGTGCACCAT ATGCGGTGTG AAATACCGCA CAGATGCGTA
4741 AGGAGAAAAT ACCGCATCAG GCGCCATTCG CCATTCAGGC TGCGCAACTG TTGGGAAGGG
4801 CGATCGGTGC GGGCCTCTTC GCTATTACGC CAGCTGGCGA AAGGGGGATG TGCTGCAAGG
4861 CGATTAAGTT GGGTAACGCC AGGGTTTTCC CAGTCACGAC GTTGTAAAAC GACGGCCAGT
4921 GCCAAGCTTT ACACTTTATG CTTCCGGCTC GTATGTTGTG TGGAATTGTG AGCGGATAAC
4981 AATTTCACAC AGGAAACAGC TATGACCATG ATTACGAATT CGGCGCAGCA CCATGGCCTG
5041 AAATAACCTC TGAAAGAGGA ACTTGGTTAG GTACCTTCTG AGGCGGAAAG AACCAGCTGT
5101 GGAATGTGTG TCAGTTAGGG TGTGGAAAGT CCCCAGGCTC CCCAGCAGGC AGAAGTATGC
5161 AAAGCATGCA TCTCAATTAG TCAGCAACCA GGTGTGGAAA GTCCCCAGGC TCCCCAGCAG
5221 GCAGAAGTAT GCAAAGCATG CATCTCAATT AGTCAGCAAC CATAGTCCCG CCCCTAACTC
5281 CGCCCATCCC GCCCCTAACT CCGCCCAGTT CCGCCCATTC TCCGCCCCAT GGCTGACTAA
5341 TTTTTTTTAT TTATGCAGAG GCCGAGGCCG CCTCGGCCTC TGAGCTATTC CAGAAGTAGT
5401 GAGGAGGCTC GAGGAGCTTG G
//
```

Figure 23. The nucleic acid sequence of the Lama2/APPA transgene used for the generation of transgenic mice and transgenic pigs (SEQ ID NO: 7)

```
LOCUS       transgene   17732 bp    DNA             SYN         14-APR-2000
DEFINITION  Lama-appA cut XhoI..20623 to NotI..17732
ACCESSION   transgene
KEYWORDS    parotid secretory protein; acid glucose-1-phosphatase; appA
            gene;
            periplasmic phosphoanhydride phosphohydrolase; artificial
            sequence;
            cloning vector
REFERENCE   1  (bases 1 to 17732)
AUTHORS     Golovan, S., Forsberg, C.W., Phillips, J.
JOURNAL     Unpublished.

FEATURES
DEFINITION  M. musculus Psp gene for parotid secretory protein.
   ACCESSION  X68699
   VERSION    X68699.1 GI:53809
   SOURCE       house mouse.
   ORGANISM   Mus musculus
      Eukaryota; Metazoa; Chordata; Craniata; Vertebrata; Mammalia;
      Eutheria; Rodentia; Sciurognathi; Muridae; Murinae; Mus.
      REFERENCE    1  (bases 3777 to 5332;)
      AUTHORS    Svendsen,P., Laursen,J., Krogh-Pedersen,H. and Hjorth,J.P.
      TITLE      Novel salivary gland specific binding elements located in
            the PSP proximal enhancer core
      JOURNAL    Nucleic Acids Res. 26 (11), 2761-2770 (1998)
      MEDLINE    98256451
      REFERENCE    2  (bases 7147 to 12653; 13952 to 17731)
      AUTHORS    Mikkelsen,T.R.
      TITLE      Direct Submission
      JOURNAL    Submitted (07-OCT-1992) T.R. Mikkelsen, Department of
                   Molecular Biology, University of Aarhus, CF Mollers Alle
                   130, 8000 Aarhus, DENMARK
      REFERENCE  3  (bases 7147 to 12653; 13952 to 17731)
      AUTHORS    Laursen J, Hjorth JP
      TITLE A cassette for high-level expression in the mouse salivary
glands.
      JOURNAL    Gene 1997 Oct 1;198(1-2):367-72
      MEDLINE    9370303

FEATURES                Location/Qualifiers
                  source          1.to 12653; 13952 to 17731
                 /organism="Mus musculus"
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                 /clone="Lambda YP1, Lambda YP3, Lambda YP7"
                 /clone_lib="Lambda-PHAGE (Lambda L47.1)"
                 /germline
                 /note="Allele: b"

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                 regulatory region"
      enhancer    7147..8724
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Figure 23 (continued):

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                /note="exon b fused with exons h and i"
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                /function=" consensus sequence for initiation in higher
                eukaryotes "
     misc_feature      13952-13965
                /function=" M13mp18 polylinker"


DEFINITION  E. coli periplasmic phosphoanhydride phosphohydrolase (appA)
gene,

     ACCESSION   M58708 L03370 L03371 L03372 L03373 L03374 L03375
     VERSION     M58708.1  GI:145283
     SOURCE            Escherichia coli DNA.
     ORGANISM    Escherichia coli
           Bacteria; Proteobacteria; gamma subdivision;
     Enterobacteriaceae;
           Escherichia.

REFERENCE   1  (bases 12653..13951)
     AUTHORS   Dassa,J., Marck,C. and Boquet,P.L.
     TITLE     The complete nucleotide sequence of the Escherichia coli
               gene appA reveals significant homology between pH 2.5
               acid phosphatase and glucose-1-phosphatase
     JOURNAL   J. Bacteriol. 172 (9), 5497-5500 (1990)
     MEDLINE   90368616

FEATURES                 Location/Qualifiers
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                         phosphohydrolase"
                         /protein_id="AAA72086.1"
                         /db_xref="GI:145285"

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Figure 23 (continued):

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ELKVSADNVSLTGAVSLASMLTEIFLLQQAQGMPEPGWGRITDSHQWNTLLSLHNAQF

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       61 ATCTAAACTA ATTAATTAAT CCCTCACCCG CAAATCTTTC AGTCACTAAG TTAGCACGAT
      121 TGTTGAACAA GTTCTCCAAA GGAGAGATAC AGATGAGTGC GTATAGGGTG GACCTGGCTG
      181 CTGAGGAGAC ACCTGCATCT GACTAAGAAG AGCCACGGTG TTAGTTGAAT GGTGTGGAGT
      241 AGGGTGGTTC TGTGGGACAG TAGAAAATCG AGAGGCATGT GCCGTTTAGT GAACTGATGG
      301 AAGCTACCCC AAACGACAGA GATTGTCAGT CAGGCCAATC CGTTTCGAGT TTGATGGGCA
      361 GCCGGACAGT GAGACAGACA CACCTACTCA GTTGGAGGAA GGATGAGAAC AATGGCCAGC
      421 AGGGATTGAG AGACCCTGAC AGGCGCAAGG CCCTAACACA CACACCTACC ACCTCACTTG
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      541 ACAGCATAAT AAGCAGAGTG TACTCTGATT GGAGAACTTT AATGTGTTTC ATTCAGTATT
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      781 GGACAATATA TATTTAGAGA AAGATGGTTA GCTGTCAGAA AAATATGCAA ATCAAAATCA
      841 CACCAAGACT GCAGCACACC CCTGTCAGAT GGCTGTGATC AAGAAAATAA ATGACAATGA
      901 GTGGTGGTGA AGATGTACTA AAGGGAAACA CACACACACA CACACACACA CACACACACA
      961 CACACTGGAG CAACCACTGT GGAAATCAGT ATGAATGGTC CTCAAAAACC TGAAGATAGA
     1021 GCGGGGCGTG GTGGCATACA CTTTTATTCC CAGCACTGGG GAGGCAGAGG CAGGTGGATC
     1081 TCTGAGTTCC AGGCCAGCCT GGTCTATAGC ACAGGTTCTA GGACAGCCAG GGCTACACAG
     1141 AAAAACCCTG CCTTGATTAA ACCAAACCAA ACCAAACCAA ACCAAACCAA ACCAAACCAA
     1201 ACCAAACCAA ACCAAACCAG ACCAAACCAA AACACTGAAG ATAGAACTTC AGTATTCCAT
     1261 TCCTAGATAT ATACCCAATG GAGACTAAGT CAGCAAGACA CCTGCACAGC CATGTTCACT
     1321 ACTACACTGT TCACCACAGC CAGGCTGTGG AACCAGCCTG AGTGTCCATG ATAAATGAAT
```

Figure 23 (continued):

```
1381 GGATAGGTAA CTTTCAAGGT AAATGGACTC TGCTGTGTAC ATGCCTCACA TTCTGTTTAT
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1501 TGAAGATACT ACACTGGTCC CCACAGTTTA CACTTTTATC AGCAGTGAAT AAGGGTTCCT
1561 CTATCCTTAC CATCATTTGT TGTAATTTTT CTTGATGACC CTCTTTCTGA CAGGGATAGG
1621 ATGTAATATC AGTGTGAGGA AGTACAACTT GTTTTCTAAG TATTTATTGG CCCCTTGCAT
1681 TTCTTCTTTT GAAAACTGTC GGTTCCTGAC ATCTGCTCAG GTATTCATTG GATGTTGTTT
1741 CTTTGGTGTT TGAGTTCTTA TGAATTCTAG ATGTTAAATC CCTGCCTGTG GTTCTCTCCC
1801 ATTCTGTAGG CTGCCTCCTC ACCCTGGCAA TTGTTGTCCT TGTTTTGCAG AAACTTTTGA
1861 CTTCATGGAA TCTCATTTGT CAGTTTTCCC TCCTCTGCTA TAGCCTGAGC TAATGCACTG
1921 GTTTTTACAG AGCCCTGGTC TATGCCTTTA TCCTCCTCTG GCAGCTTCGG AGTTTCATTT
1981 CTTACATTTA GATCTTTGAT CCACTTTGAA CAAGTTTTGG AGCAGGGTGA GAGATACGAA
2041 TCTAGTTCCA TTCTTCCATA TGTGATCCTA GTTTACATAG CATCGTTGGT TGAAGAGGTT
2101 TTATTTTATT TTTAAATAAT GTGTCATAAA AAACGAGGTG GTTGTAGCAG TGTGGATTTG
2161 TTTCTTTGTC CTTTGATCTA CAGGTCTTGT TTTGTGTCAG TCTCATGATG TTTTATTGCT
2221 ATGGCTCTGT CATACAGTCT GAGGTCAGGT ATTGTGATAT ACCTTCAGTA TTGCTCCCTC
2281 AGACTCAGGT TTGCTTTGGC CAGGAGTCAT CTTACTCAGT GCTCTTAGAG CTCCCCCAGC
2341 ATGTAGCTGC TACTATTCTT AGTTGATAAA TCAGGAAACT GGGGCTCAGA GAGATTAACT
2401 GTCTTGAACT ACTTCTGGGG AGGTGAAACG TGGAGACACT AAACTGTGTT TACCCTGTAC
2461 TGCTCCAGTA GCTGTCGGGT GCTGGGCTAC AGCAAAGCAC CTATACTATA TATTACTCAG
2521 GAGGTGGAAA AACTCAGCCT CCCTTGGGGT TCCCAAGCTC CCAGGTGTCC AGTCACTGCT
2581 GGAAACCTCA TGGAGTCTGA AAGGAAGGGT TGAGGGTACA TGGGGCAGCG ATGAGGAGCC
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2701 GAACAGAGTT GGGATGTCCA TGGACCTGTG ACAAGGCCAG GGCCAGGGGG AGGATAACTC
2761 TGGCTTTACT AATTTGCGAA AGTCCTTAGC TTAGCAGCAG TTGTCTGGGA GCACAGAGGG
2821 GCCTTCTGTA AGAGGCTCAG GCAGTGCCGC TCTGTAGGCG AAGGTCTTCT CCATGTTCCC
2881 CATGGTGGTT CTTGATGAAA GAGACAGTCC TTGGCTCCAA ACTGGTTTAT TGATTGTTCA
2941 TTGTGGAAAA TGGGTGCACA CCACCTTCTC AGGGTGGACC AGAGATCAAA TACCTTTTGC
3001 AGGGAGGAAT ATCTGGGAAG GGACGCTTAC TGGCTAAACC CTCAGGGCCT CTAGATACAT
3061 CATTAGCATG GAGAACTCTG TTCTGGGCTA CATGACCACA GGCCACATTT CCACAAGCCA
3121 CATGTGGGAA GTGTGGCACA TGTTCTAGGC CAGGAATCTG GTAGGGAGCG TGGAGCCACC
3181 TACCATCCCA GGTGGGTGCC TGGGTGCCAG GGACCCTGAA CCCGCTCAAC CTTACCAAGT
3241 TTCCTGGCAG GGTCCACTGT CCTACACAGA AGCTGGAGGA GGTGTGAGGG TTGTGTCTTT
3301 GTGGAATGTC CCATGCTGCT TGGGGCTCAG TTTCTCCACC TGTACCTCAT TGGTTTGGGT
3361 ATAAAAAGTG GGGATACTTT ATTATTCTCT GACTCGGTCC TGAGGAAAAA GCATCGTGGC
3421 AGTCCAGGAA CCACACCCTG AGGTTCCTGC ACTGAAGGGA CTCCCTAAGT CTCTGGAGTC
3481 TCTCCCCTTC ACAGAGCTGC CAAAGTCTAG GTTCTTTTGA GGATAACAGA GCCATGCTTG
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4141 GGTACACCCA CTCCTCTGCC TGTGTGGTTC CTGCAGTCAG TCCTGCAGAC AGGCCCTCAG
4201 TGGGTCTTCC ATGGGCAACA CGCAGAGGGA GGCAATGGAT GGGAATACCC ACACCCTGGT
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4321 CTCTGCAGGA ATCATATCTT CATATTGGCC CACAGGTGTT CTCCTCACCC TAGCTATGAT
4381 GTTTACTTTA GAGTGACCTT AGCAGGGCTG GTGGGAATGA GTTCTAGAAG GCTCACGGAG
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4561 GAGTAAATAT GGTCCTGAAG ATTTCCTTTG AGTGCCCAGA ATCCATGACA TTTCAAGAGC
4621 CCTCTTTGTA CCTTAAGTCA TTTGGGGTTG TATCTTCTGC TTGATGTATG TGTGTGTGTT
4681 TATCAAAGAG TGAGATGGTT ACATAAGAGG TGCTCTAAAG GACAGAGAGG ATTTGCAATT
4741 GTGGCATGTG ACATCCTCAG GCCTTGCTCT GGTGCCAGGA GGAACTGATG CAGAAAAGAG
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```

Figure 23 (continued):

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4981 CACCAGCTCC TGACCACCCG GTCAGCCCAT GTGCTTATTC CATAGCTTTC TTTTGCTATG
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5401 TTTGACATAT TTAAACAGAG CACAACTTTG GGAAAAAGTT TTCTTATGAA AATTATCACA
5461 ATAAAGCTTA AGGCATGACT ACATTAAAAT GCCTTTGCAA AGTATATGTG CCCTCTTCCA
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6481 GTTATAAACC AAATCTTAGG GAGTCAGGAA GAGCACAGAG GAGCTCAACC AACTGACCAC
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6601 AATAAACAGG CAGGACCTGT GGATGACATG GAGAGCATAG GGACCCTGGG TCCAGCCTTT
6661 AGCACCTGCA CTCTCAGGAT ACTCCACCAT TGTGTCTTAG AGAGCCTAGG GATACTGGGT
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7141 CTTCGAAAGC TTTCAGCAAA TGCTCAGGGA ACATGCCACT AACAAGAAGA TGCAAATTCC
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7261 TCTGTGTAAT CCGGATTGAC AGGGCTCAGA ACAATGTTTT GTTTTTAAGG TTTATTTATT
7321 TTAGGTGTTA GTGTCTTTGC TTGCATGACC TTATGTGCAT CATGTGTGTG CAGGTTCCTG
7381 ATGACAGTAG AGGAGGGCTT TGAATCCCTG GGGATAGGAA GTTACAGGAA ATTATAAGCT
7441 GCTTTGTGGG TCTTCTAGCT TTCCCAACAG AAGTGAATGC TCTTCACCAC TGAGCCATCT
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7981 CCAGCCTCTC TGGTCCTAGT AGCTTTTTCC AAACAGGAAT CTGAGTGGTG ACAGGGAACA
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8161 ACTGGAGATT TTCAATGAGA AATTTATCCC ACGGCCCATA TGGCCCCATC CTTTTGTCTC
8221 CAACAGCCAA GTATTTTCCA TTAGAGGAGA CTTCCTGTAC ACTTGATGGA TGCTCATTCC
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```

Figure 23 (continued):

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 8341 CAACAAGGGC CCTCTATGTT TGCTATGTAA TGTAATGTCA GACATTGTCA GGAGTGTCCG
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 8461 GATAACGAGG AGGTAAGCTG CAGTTCCCAG TCTCACTTCA CAGAGGAAGA GATAACCCCA
 8521 TCCCAGGTCA TGTAGCCAGC AGTGGAAAGA ATGAGGATTT GAACTCAGGT CTTCCAAGTC
 8581 CCATTGATAG CATCTCCTCA CAAGTCCCTT GCCACCCTCA CGATGCCTTA GACACTTGCC
 8641 TGCCCTTTAT ACTAAGGAGA TGCAGGTACA AGGGGTTTAC CCATGTAGCA GCTGAGGCAG
 8701 CTGGGGATAG ATACCAGCAG CAGGCCTGAT GTCACCACTC TAACTCCAGC ATCCCCAGTC
 8761 TGTGTTCCTG GAGTGTGAAA ATCCCTACTT AACAAGATTG TGCAACAGTC CTTGGCTCTG
 8821 TGACCCATAG CTGGAAACAG GATTCTCATT GATTTGTGGA ACATGGTGGC AGCCAGCCAA
 8881 AAAGAGGGTC TGCATACAGA AGACACGTGT GGCAAGGCCA CAGCAGACTC TGACTACCTT
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 9061 GAGACCCATC CTACAGGCAA GCATCAATTC CTGACACTAC TAATGATACT CTGTTATGCT
 9121 TGCAGACAGA AGCCTAGCAT AACTATCCTC CGAGAGGTCC ACCCAGCAAC TGACTGAAAC
 9181 AGAAAAAGAT ATCCACAGGC AAACAGTGGA TGGAGGTCAG GGACTATTAT GGGAGAGCTG
 9241 TGGGAAGGAT TAAAAACCCT GAAGGGGATA GGAACCCCAC AGGAAGACCA ACAGAGTCAA
 9301 CTAAGAGACC TGTGGGAGCT CTCAGAGACT GAGCCACCAA CCAAAGAGCA TACACAGGCC
 9361 GGTCCGAGGC ACCTGGCACG TGTGAAGCAG ACATGCAGCT CAGTCTCCAT GTAGGTCCTC
 9421 CAATAAGCGG TAGCCTGACT GCAGTATCCA ATCCCCAACA GGGCTGCATA GTCTGGCCTC
 9481 AGTGGGGGAG GATGCCCCTA ATCCTGCAGA GACTTGATGA GTGGAGAGCT ATCCAGGGGG
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 9601 GGACAAACAA GAACCTCAAA TAGGTCAGGC CCTAAAGGCT TGCTAAGTAG CAGTGGCCCA
 9661 GCTCTGTCCT GTTCCTCAGC CCAAGGCTCA GCTCCCACCT GTTTCTGTGT TTTTCTGGCT
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 9841 CCCAATCACT GGATTTCTAC CCTTGCACAA CCTTGACAGC TGAGGGCCTC TCAGAAACCT
 9901 ATTTCTTCCA CTGAAAAATG AGACTCTCAA ATGAACGTCG TGACAATCAT CAGGCTTATT
 9961 AAAGAGGTGT ATCTAACCTG AATGGCAAGC AGACAGCAGG CAAATGTCTG TATCAACCTC
10021 TAGGAAGGAC AAGAACTGCT CACTGCTGCC CCCCAGGAGG CCATTTGCTG AAACAGCTGC
10081 TCTCCTGCTG GTGCACAGGC CCTGCCTTCT CATTGCAGCC ACAGCCCCTT CCTGTCTGAA
10141 CCTCCTGTCA GGTCACTGGG AAACAGATCA AGATGGAACA GGACAGCTCC TGATGGTAAA
10201 TAAAAAACAG TGGTCATGGC TATTCATAGG GGTTTATGCT TCTTCAGTCC ACACTGTGAA
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10561 AACTATCAGG CGTTGTCCCC TCCTACTTTT CCTCGGACTC TGGAGAACAA ACATGGGTCC
10621 TTATTCCAGG GGAGCAAGTC GCTGTTGGCT GACACATCTT GCTCACATAC ATTTTACCTA
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10981 TCCCTTTGGA CCACAGACAT CCTGGCTTGT GAATTCACAT GTCATCTACT GCTAATCCAT
11041 TGGTAGTATG TGGCTCACAG AGACACACTA CAGTCATGGC CAATGTCAAG GTAGGACAGA
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11641 CCTGGGTGAC CTTTGGGTTG GAATATCTTG GCTAACCTTA GATATTTTCT TTGGAGTATC
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```

Figure 23 (continued):

```
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Figure 23 (continued):

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Approved for use through 10/31/2002. OMB 0651-0032
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE

DECLARATION (37 CFR 1.63) FOR UTILITY OR DESIGN APPLICATION USING AN APPLICATION DATA SHEET (37 CFR 1.76)

As the below named inventor(s), I/we declare that:

This declaration is directed to:

☐ The attached application, or

☒ Application No. PCT/CA00/00430, filed on April 20, 2000,

☐ as amended on _____ (if applicable);

I/we believe that I/we am/are the original and first inventor(s) of the subject matter which is claimed and for which a patent is sought;

I/ we have reviewed and understand the contents of the above-identified application, including the claims, as amended by any amendment specifically referred to above;

I/we acknowledge the duty to disclose to the United States Patent and Trademark Office all information known to me/us to be material to patentability as defined in 37 CFR 1.56, including material information which became available between the filing date of the prior application and the National or PCT International filing date of the continuation-in-part application, if applicable; and

All statements made herein of my/own knowledge are true, all statements made herein on information and belief are believed to be true, and further that these statements were made with the knowledge that willful false statements and the like are punishable by fine or imprisonment, or both, under 18 U.S.C. 1001, and may jeopardize the validity of the application or any patent issuing thereon.

FULL NAME OF INVENTOR(S)

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Signature: X Cecil Forsberg Citizen of: Canada

Inventor two: Serguei Golovan

Signature: X _____ Citizen of: Canada

Inventor three: John P. Phillips

Signature: X _____ Citizen of: United States

Inventor four: _____

Signature: _____ Citizen of: _____

☐ Additional inventors are being named on _____ additional form(s) attached hereto.

Burden Hour Statement: This collection of information is required by 35 U.S.C. 115 and 37 CFR 1.63. The information is used by the public to file (and the PTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This form is estimated to take 1 minute to complete. This time will vary depending upon the needs of the individual case. Any comments on the amount of time you are required to complete this form should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, Washington, DC 20231. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. SEND TO: Assistant Commissioner for Patents, Washington, DC 20231.

Initial Information Data Sheet

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1-00

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State or Prov. Of Residence::	[include this only if different from postal address]
Country of Residence::	[include this only if different from postal address]
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Country of Residence::	[include this only if different from postal address]
Citizenship Country::	Canada

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State or Prov. Of Residence::	[include this only if different from postal address]
Country of Residence::	[include this only if different from postal address]
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Application Information

Title Line One::	TRANSGENIC ANIMALS EXPRESSING SALIVARY
Title Line Two::	PROTEINS
Title Line Three::	
Total Drawing Sheets::	58
Formal Drawings?::	Yes
Application Type::	Utility
Docket Number::	6580-270

Representative Information

Representative Customer Number:: 001059

Continuity Information

This application is a::	371 of
> Application One::	PCT/CA00/00430
Filing Date::	April 20, 2000
which is a::	Non Prov. of Provisional
>>Application Two::	60/130,508
Filing Date::	April 23, 1999

which is a::
>>>Application Three:
Filing Date::

which is a::
>>>Application Four:
Filing Date::

Prior Foreign Applications

Foreign Application One::
Filing Date::
Country::
Priority Claimed::

SEQUENCE LISTING

<110> University of Guelph
Forsberg, Cecil W.
Golovan, Sergeui
Phillips, John P.

<120> Transgenic Animals Expressing Salivary Proteins

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<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: R15/APPA +

intron plasmid with pBLCAT3 vector

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<211> 4060
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: R15/APPA +
intron transgene

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<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: R15/APPA
plasmid with pBLCAT3 vector

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<213> Artificial Sequence

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<223> Description of Artificial Sequence: R15/APPA
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<211> 5421
<212> DNA

<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: SV40/APPA + intron plasmid

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